STUDIES ON OVARIAN AND UTERINE FUNCTION IN THE MARE

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The submitted collection of papers represents my work in the area of equine ovarian function and dysfunction. During spring transition, the endocrinological events responsible for recruitment of large anovulatory follicles appear to resemble recruitment of preovulatory follicles in the natural breeding season. These large follicles contain only low concentrations of progesterone and oestradiol. They have low expression of mRNA encoding steroidogenic enzymes, have poor development of the theca interna and are poorly vascularised. These follicles are therefore showing signs of atresia while they are actively increasing in size. In preovulatory follicles, concentrations of inflammatory mediators increase as ovulation approaches, and fluid from preovulatory follicles is chemotactic for leucocytes. Intrafollicular treatment with indomethacin delayed ovulation which supports the central role for inflammation in the ovulatory process. It is also likely the matrix metalloproteinases are involved in the profound tissue remodelling that occurs around ovulation. Control of follicular growth has been studied and equine follicles are dependent on gonadotrophin stimulation when they reach 10 mm in diameter and on LH stimulation for final growth and maturation.

The equine CL is dependent, at least in part, on trophic support by LH and luteal cells bind LH *in vitro*. Steroidogenesis in the CL varies before and after development of the endometrial cups in pregnancy. StAR protein increases after endometrial cup formation, allowing greater mobilisation of substrate for steroid synthesis. Although $P450_{arom}$ is consistently present in the equine CL, $P450_{C17}$ increases after the endometrial cups form, allowing oestrogen synthesis by the CL in the pregnant mare. The cell types in the equine CL appear to cooperate in steroidogenesis, with $P450_{C17}$ located in small luteal cells, and $P450_{arom}$ in large luteal cells. Maintenance of the CL in early pregnancy appears to be caused by the inhibition of endometrial PG synthesis by the conceptus, although the conceptus itself produces PGs. The demise of the CL involves apoptosis and is preceded by a decrease in angiogenesis. The immune system appears to play a significant role, with an influx of CD8+ lymphocytes into the CL prior to functional regression. At this time also, the CL is producing substances chemotactic for immune cells.

Endometritis is the commonest cause of subfertility in mares. Mares prone to endometritis have low myometrial contractility compared with reproductively-normal mares and this appears to be due to a defect in the uterine oxytocin receptor or post-receptor mechanisms. The uterus has also been shown to be a source of oxytocin with the hormone located in secretory vesicles of the epithelium of the lumen and superficial glands. In mares with chronic uterine infection, chemotactic factors are present in uterine fluid that enhance the migration of neutrophils into the uterine lumen. However uterine fluid from infected mares interferes with phagocytosis and the opsonic activity of specific antibodies in the fluid is lower than that from reproductively-normal mares. Apart from a possible relative deficiency in numbers of macrophages, no abnormalities were detected in the cellular immune system in mares prone to endometritis.

CONTENTS

DECLARATION

The vast majority of scientific, and particularly veterinary, research is a team effort. During my time at Bristol, when I was undertaking my PhD, I was given support and encouragement by my supervisors Professor John Bourne, Professor Chris Stokes, and Dr John David. I was also given help and guidance by other members of the large veterinary immunology research group that was active at Langford at that time. When I moved to the University of Pennsylvania, I was given assistance with the clinical component of my research by Dr Patricia Sertich. Heidi Zanecosky provided technical input and was supervised by myself in performing the assays. Since my arrival in Edinburgh 11 years ago, I have had a series of clinical assistants and PhD students whom I have supervised. Technical assistance in Edinburgh has been provided mainly by Sheila Thomson. I have collaborated with scientists at the Roslin Institute, and at the MRC Centre for Reproductive Biology, as well as the Department of Obstetrics and Gynaecology.

Detailed below are the collaborators I have worked with for the research contained within this thesis:

1. Scientific collaboration

Professor John Bourne, University of Bristol School of Veterinary Science
Professor Chris Stokes, University of Bristol School of Veterinary Science
Dr John David, University of Bristol School of Veterinary Science
Dr Patricia Sertich, University of Pennsylvania Veterinary School
Dr Katrin Hinrichs, University of Pennsylvania Veterinary School
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INTRODUCTION

After graduating for the University of Glasgow Veterinary School in 1978, I was awarded a Faculty Scholarship and successfully completed a Master of Veterinary Medicine in 1979. This degree involved performing a research project on factors affecting cyclicity in dairy cows. My main supervisor, Dr Colin Munro, instilled a sense of enthusiasm for research in the area of clinical reproduction that has stayed with me over the ensuing 23 years. This early introduction to clinical investigation resulted in me remaining in the area of bovine reproduction research, first at the MAFF Cattle Breeding Centre, Shinfield, and then at the AFRC Institute for Research on Animal Diseases, Compton, until 1984. Over this time I published 16 papers on bovine reproduction. I have maintained an interest in cattle since then and have published a further 15 papers. However in this thesis I have included only work performed since 1984 in the horse, my species of choice.

In 1984 I joined Professor Bourne's large veterinary immunology group at Bristol as a PhD student to perform an HBLB-funded project on immune defence mechanisms in the equine uterus. As I had already spent 6 years in research, I was able to "hit the ground running" with techniques learned at Glasgow, Shinfield and Compton. John David worked beside me initially to get me up to speed on clinical equine reproduction and I spent a highly productive and happy three years at Bristol studying cellular and humoral responses in the endometrium.

In 1987 I was recruited to the University of Pennsylvania School of Veterinary Medicine as an Assistant Professor in Equine Reproduction. Here I spent 50% of my time in the clinic and learned many new clinical techniques from Katrin Hinrichs, Pat Sertich and Bob Kenny. My research here was focussed on ovarian activity and maternal recognition of pregnancy. In 1990 I submitted a thesis to the RCVS for my Fellowship entitled

"Studies on Ovulation in the Mare". This work had involved Pat Sertich and myself in many night-time sessions scanning ovaries and aspirating follicular fluid from mares.

In 1991 I was recruited to Edinburgh by Richard Halliwell and my research became more supervisory in nature with a series of PhD students whose projects included reproduction in cattle, goats, horses and turkeys. I also established contact with my many collaborators mentioned earlier, as well as Bob Webb with whom I co-supervised a student on bovine reproduction. In the mare, the areas that I concentrated on were ovarian function and endometritis.

The area of equine reproduction, on which this thesis concentrates, represents a small community of dedicated clinicians and researchers. An International Symposium on Equine Reproduction is held every four years and reinforces the collegiality prevalent among these veterinarians and scientists. All are connected through the intrinsic difficulties of their research area. Namely: i) the expense of purchasing and maintaining horses, ii) the dearth of abattoir material, iii) the work-intensive nature of sample collection from such large animals, iv) the paucity of reagents specifically available for the horse, and v) the lack of research funding as the horse is not generally classed as an agricultural animal, is not a useful model for human infertility, and reproduction research is not eligible for welfare charity funding.

The research included in this thesis comprises two inter-related areas:

1. Ovarian physiology in the mare a) Studies on follicular activity
   b) Studies on the corpus luteum
2. Endometritis in the mare

Section 1: Ovarian Physiology in the Mare

a) Studies on follicular activity

1.1 Transitional breeding season

Most mares exhibit cyclical ovarian activity only in summer, during periods of prolonged daylength (21/21 of the pony mares studied). In the period between winter anoestrus and regular cyclicity, many mares go through a transitional period of irregular ovarian activity. The mares may start to grow small follicles which regress when they are less than 30 mm in diameter. They can then progress to having cyclical growth and regression of large dominant ovarian follicles at approximately 10 day intervals (11/16 (69%) of mares studied). On average our mares had 3.6 $\pm$ 0.6 of these anovulatory follicle waves before one follicle went on to ovulate (Ref 1.1). The median date of first ovulation was May 3. This period of spring transition is characterised by low concentrations of circulating LH which increase just prior to first ovulation. The low circulating concentrations of LH probably contribute to low levels of expression of mRNA encoding steroidogenic enzymes ($P450_{SCC}$, $P450_{C17}$ and $P450_{arom}$) in these follicles (Ref 1.2) and poor development and vascularisation of the theca interna (Ref 1.3). The low vascularity and proliferation of endothelial cells was probably directly related to the low levels of vascular endothelial growth factor (VEGF) in the theca of transitional follicles (Ref 1.3). This resulted in these large transitional follicles being steroidogenically incompetent and producing only low concentrations of oestradiol and progesterone (Ref 1.4). These findings are similar to those in cyclic follicles destined to become atretic. Therefore even

though the transitional follicles are increasing in size, they are showing signs of atresia. Changes in follicular expression of mRNA encoding insulin-like growth factor binding protein-2 appeared not to be related to the low levels of steroidogenesis in transitional follicles (Ref 1.5).

Although follicular oestradiol production is low at this time, there is still a significant elevation in circulating oestradiol which, although small, can produce ultrasonographically-visible uterine oedema (Ref 1.1). This suggests that in the absence of progesterone, the uterus is highly sensitive to very low concentrations of oestradiol. It also dispels the commonly held belief that uterine oedema is not present until the first ovulatory follicle of the breeding season. Interestingly, recruitment of these dominant anovulatory follicles appears to be similar endocrinologically to the natural breeding season, with each anovulatory wave being preceded by a peak in FSH, and then an increase in circulating bioactive inhibin A which likely feeds back negatively on FSH secretion (Ref 1.6).

1.2 Ovulation

The process of ovulation resembles an inflammatory event. Concentrations of inflammatory mediators increase in follicular fluid as time of ovulation approaches, and there is an influx of immune cells. In the mare, as the follicle matures towards ovulation, intrafollicular concentrations of arachidonate metabolites, as well as testosterone and

progesterone, increase (Refs 1.7, 1.8). Furthermore, intrafollicular administration of indomethacin, an inhibitor of cyclooxygenase, delays ovulation/luteinization (Ref 1.8). A leukotactic factor has been demonstrated *in vitro* in preovulatory follicular fluid which might act to increase numbers of inflammatory cells in follicles prior to rupture (Ref 1.9). Although the inflammatory cascade may be required for the events leading up to follicle rupture, after ovulation suppression of the immune response may be necessary to allow fertilisation of the oocyte by antigenically-foreign spermatozoa. Preovulatory fluid is increasingly suppressive to lymphocyte blastogenesis as ovulation approaches, which might act to inhibit an immune response within the oviduct to the zona pellucida around the time of ovulation (Ref 1.10).

The preovulatory follicle in horses and ponies grows to an average of 42 mm in diameter before ovulation (Ref 1.11), and because they ovulate at only one point on the ovarian surface, the ovulation fossa, the preovulatory follicle migrates through the stroma within a few hours of ovulation. We have shown by zymography, reverse zymography, and immunohistochemistry that matrix metalloproteinases (MMPs) and their tissue inhibitors, the TIMPs, are probably involved in this process of migration through the equine ovary. The MMPs are key enzymes in the degradation of extracellular matrix during tissue remodelling. MMP-2 and –9, and TIMP-1, -2, -3, and –4 are present in the granulosa and theca cells of the equine follicle as well as the ovarian stroma and MMP-9 increased with follicular development (Ref 1.12).

1.3 Follicular growth and viability

The ability to induce mares to ovulate multiple follicles has obvious advantages in techniques such as embryo transfer. However, it has proved very difficult to superovulate mares. Administration of crude equine pituitary extract is successful in increasing ovulation rates, but is not commercially available. Ovulation rates were increased by inhibiting follicular growth by administration of oestrogen and progesterone, and then stimulating a cohort of follicles to grow using a GnRH agonist (Ref 1.13). However the increase in ovulation rates was slight and fewer embryos were recovered per ovulation. That study demonstrated the stimulatory effect of gonadotrophins on maturing equine follicles, later confirmed by treatment with a GnRH antagonist (Refs 1.14, 1.15) and agonist (Ref 1.16). This latter study showed that equine follicle growth is independent of gonadotrophin stimulation until the follicles reach 10 mm in diameter.

Extraction of DNA from follicles followed either by agarose gel electrophoresis or by 3′-end labelling, demonstrated that smaller follicles are less likely to be healthy than larger follicles. Fifty per cent of follicles < 20 mm showed evidence of apoptotic cell death, whereas no follicles > 30 mm had evidence of apoptosis. We were also able to correlate the presence of granulosa cell apoptosis with oocyte quality and showed that apotosis was associated with cumulus expansion (Ref 1.17).

Research into *in vitro* manipulation of equine oocytes has mainly been hampered by lack of a reliable supply of a large number of healthy oocytes. However, ovaries from young mares contain approximately 1,000 preantral follicles which represents a rich source of biological and genetic material. We showed that intact preantral follicles can be successfully isolated from equine ovaries by enzymatic digestion (Ref 1.18). Future studies will further refine these techniques including assessment of oocyte quality and follicle viability.

1.4 Clinical ovarian problems

Granulosa cell tumours are one of the commonest neoplasms in mares and represent the commonest ovarian tumour (reviewed in Ref 1.19). However the transrectal ultrasonographic appearance of these affected ovaries is not diagnostic for this sort of neoplasm (Ref 1.20). Approximately 50% of mares with this pathology have elevated circulating concentrations of testosterone (Ref 1.20), but a definitive test for granulosa cell tumours was needed. In a clinical study we were able to demonstrate that mares with granulosa cell tumours produce high concentrations of the inhibin isoforms, inhibin pro- and -αC (Ref 1.21). The only normal physiological state in the mare in which similar concentrations might be reached is around ovulation. Although there is now a demand for inhibin assays by veterinarians, none is commercially available in the UK.

b) Studies on the corpus luteum (CL)

1.5 Corpus luteum support

The physical removal of follicular fluid from the equine preovulatory follicle in itself results in formation of a functional CL (Ref 1.22), suggesting that follicular fluid contains a luteinization inhibitor. Maintenance of normal function of the CL in the mare appears to rely on provision of a pulsatile pattern of secretion of LH, and treatment of mares with a GnRH antagonist resulted in attenuation of CL function (Ref 1.23). Although we failed to demonstrate a tight temporal asociation between LH and progesterone in the luteal phase, treatment of dioestrous mares with hCG increased circulating concentrations of progesterone, suggesting stimulation of progesterone production by LH (Ref 1.24). We were also able to measure LH binding by luteal cells *in vitro* (Ref 1.25), providing further evidence for the role of LH as a trophic factor in luteal maintenance. The demonstration of the presence of specific, high affinity binding sites for progesterone in the equine CL (Ref 1.26) suggested that progesterone itself may play a role in regulation of CL function. In order to investigate luteal function *in vitro*, we studied methods of preparing luteal cell suspensions and showed that enzymatic digestion substantially altered the types of cells present, with a significant reduction in the proportion of large luteal cells (Ref 1.25). It also appeared that the properties of the isolated luteal cells were impaired compared with our results in living mares. This study demonstrated the importance of isolation

procedures and culture conditions in *in vitro* studies and may explain the diversity of results obtained by other workers.

1.6 Steroidogenesis

Continuity of steroid secretion is essential in maintenance of early pregnancy in the mare. Around Day 35 of pregnancy, when the chorionic girdle cells invade the endometrium to form the endometrial cups, secondary and accessory CL develop in the mare's ovaries. Coincident with the development of the additional CL, circulating oestrogen concentrations increase. We were able to demonstrate that steroidogenic acute regulatory (StAR) protein, thought to be the rate limiting enzyme in steroidogenesis, is present in the CL of both the pregnant and the non-pregnant mare. Immunohistochemical techniques showed that StAR was confined to the large cells of the CL (Ref 1.27). Quantitation by western blotting followed by phosphor imaging demonstrated that StAR protein increased in CL recovered after Day 35 thereby enhancing the capacity of the CL to secrete steroids. Furthermore, although $P450_{arom}$ was consistently present in CL (Ref 1.28), $P450_{C17}$ increased after Day 35 of pregnancy (Ref 1.29). This indicated that the lack of availability of androgen substrate acts as a limiting factor in oestrogen synthesis by the equine CL prior to development of the endometrial cups. Furthermore we were able to show that there appeared to be cooperation between different cell types within the equine CL in steroidogenesis. $P450_{C17}$ was confined to the small cells, whereas large cells contained $P450_{arom}$ (Ref 1.30). Because $P450_{C17}$ is located in the theca interna cells of the

preovulatory follicle (Ref 1.29), and $P450_{arom}$ confined to the granulosa (Ref 1.28), we suggested that the small luteal cells may be derived from theca, and the large luteal cells from granulosa.

1.7 Maternal recognition of pregnancy

Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) from the endometrium is thought to be the principle luteolytic factor in the mare. By isolating and culturing endometrial epithelial cells and stromal cells, we were able to show that the epithelial cells were the main source of $PGF_{2\alpha}$ (Ref 1.31). Release of $PGF_{2\alpha}$ from the endometrium was inhibited *in vitro* by the presence of a 14 day equine conceptus (Ref 1.32) and the endometrium of the early pregnant mare possessed an intracellular inhibitor of prostaglandin synthesis within the isolated microsomal fraction (Ref 1.33). Equine conceptuses themselves produce prostaglandins *in vitro* (Ref 1.32). However, circulating concentrations of PGFM in mares do not increase early in pregnancy. Therefore it appears that the $PGF_{2\alpha}$ produced by the conceptus remains within the uterine lumen, perhaps inducing myometrial contractions at a time when conceptus mobility is crucial in ensuring contact of the small spherical conceptus with the entire endometrial surface. A luteotrophic role has been suggested for PGE_2 in other species, but we were unable to demonstrate a luteotrophic effect of conceptus products on cultured equine luteal cells (Ref 1.34.). The conceptus and endometrium from early pregnant mares do, however, secrete substances that suppress lymphocyte blastogenesis *in vitro* which may assist the antigenically-foreign conceptus to survive in

the maternal uterus (Refs 1.35, 1.36). It was suggested that PGE_2 in the pregnant uterus may be involved in suppression of lymphocyte blastogenesis (Ref 1.36).

1.8 Luteal regression

We showed that the equine CL itself secretes arachidonate metabolites (Ref 1.34) and suggested that the change in ratio of metabolites secreted as the CL ages may contribute to its demise. $PGF_{2\alpha}$ does decrease progesterone production by equine luteal cells *in vitro*, and increases production of leukotriene B4, suggesting a possible role for LTB_4 in luteolysis (Ref 1.37). Oxytocin is not synthesised in the equine CL (Ref 1.38) and therefore luteal oxytocin does not act as a trigger for luteolysis in the mare. The immune system appears to have a central role in CL demise in the mare and there was a selective influx of CD8+ T lymphocytes into the CL prior to functional regression (Ref 1.39). We have also shown that supernatant from CL incubations were chemotactic for neutrophils and mononuclear cells and this property was manifest just before functional luteolysis (Ref 1.40) at the same time as the ingress of immune cells.

Ultrastructural and immunohistochemical observations on the regressing equine CL showed that apoptosis was one form of cell death in this structure (Ref 1.41). One of the earliest changes observed was rarefaction of the mitochondria which occurred as early as Day 10 in the luteal phase, when progesterone production is still high. We also showed by immunohistochemistry and morphometry, that angiogenesis and its control by vascular

endothelial growth factor (VEGF) appear to be important in control of both CL growth and regression (Ref 1.42). This indicates that pharmacological inhibition of VEGF might offer an important alternative means of more precisely controlling CL lifespan in the mare.

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Section 2: Endometritis in the mare

Fertility varies markedly among mares. Apart from poor management and incorrect timing of mating, probably the most important reason for low pregnancy rates is endometritis. The multifactorial nature of the condition has resulted in subdivision of endometritis into four categories, based on aetiology and pathophysiology. These are (1) endometrosis (chronic degenerative endometritis) (2) sexually transmitted diseases (3) persistent mating-induced endometritis (PMIE), and (4) chronic infectious endometritis. These categories are not, however absolute and mares may change categories between, or even within, breeding seasons, or may fit into more than one category. The research performed by the author has concentrated on PMIE and chronic infectious endometritis.

2.1 Persistent mating-induced endometritis

Endometritis is a normal physiological event after mating, but if the inflammation persists, the resulting environment is not compatible with establishment of pregnancy. The inflammation is often accompanied by accumulation of intrauterine fluid. We have shown that 35% of mares accumulate intrauterine fluid at 12 h after insemination with frozen-thawed semen. This is reduced to 14% by 18-24 h (Ref 2.1).

It is thought that a primary defect in myometrial contractility may contribute to PMIE. Visual analysis of video recordings has been used to study myometrial contractility in

mares and we have reported that there are significant visual differences in uterine motility between reproductively-normal mares and mares which show delayed uterine clearance (Ref 2.2). Uterine contractility is under hormonal and neural control. The hormone oxytocin acts both directly on the myometrium to cause contraction, and indirectly via uterine release of $PGF_{2\alpha}$. After foaling however, concentrations of the circulating metabolite of $PGF_{2\alpha}$, 15-keto13,14-dihydro $PGF_{2\alpha}$ (PGFM), are low by Day 1 postpartum, which does not help to explain the rapid rate of uterine involution after foaling in the mare (Ref 2.3). The main source of oxytocin is probably the hypothalamo-neurohypophyseal axis. However the mare's uterus also appears to be a source of oxytocin. We have used immunohistochemistry to demonstrate the presence of both oxytocin and its carrier neurophysin in the luminal and glandular epithelium of the uterus (Ref 2.4). At the ultrastructural level, oxytocin and neurophysin were detected by immunogold labelling in the secretory vesicles of the luminal epithelium and the epithelium of the superficial glands (Ref 2.5). A large range of stimuli can cause oxytocin release in mares, the greatest being vaginal stimulation (Ref 2.6). Reproductively-normal mares and mares with PMIE had similar circulating concentrations of oxytocin after artificial insemination, however administration of oxytocin to reproductively-normal oestrous mares resulted in a markedly greater release of $PGF_{2\alpha}$ compared with mares susceptible to PMIE (Ref 2.7). This has led to the suggestion that there may be a difference in these two groups of mares at the level of the uterine oxytocin receptor, or in post-receptor mechanisms (Ref 2.7).

There is very little information on neurological control of myometrial contractility in the mare. Studies on the organisation of innervation in the normal equine uterus (Ref 2.8) showed that the equine uterus is supplied mainly by adrenergic nerves. Of the peptidergic neuropeptides, neuropeptide Y was the most abundant (Ref 2.9). Further confirmation of the importance of neurologic control of myometrial contractility was obtained by administration of clenbuterol, a β_2 agonist, to mares which suppressed uterine contractility and clearly predisposed normal oestrous mares to intrauterine fluid accumulation after intrauterine bacterial challenge (Ref 2.10).

Regardless of whether mares are bred by natural mating or artificial insemination, breaching of the cervix at breeding results in an equally intense acute inflammatory response in reproductively-normal mares (Ref 2.11). Although many bacteria will inevitably be introduced at this time, uterine inflammation can persist after breeding in the absence of bacterial contamination (Ref 2.11). Volume of the inseminate may influence persistence of uterine inflammation and larger volumes decrease the inflammatory response (Ref 2.12). In reproductively-normal mares, this post-breeding endometritis is transient and subsides within 48-72 h. However if intrauterine fluid is present at 12 h or more after mating, the mare is considered to have PMIE (Ref 2.13). After induction of inflammation, components of the intrauterine fluid comprise inflammatory mediators, neutrophils and plasma proteins including immunoglobulins, complement and enzymes, including equine α-1 proteinase inhibitor (Refs 2.14, 2.15, 2.16, 2.17). These

inflammatory components are increased within 30 min to 12 h of uterine insult (Ref 2.16) and speed of mobilisation of the phagocytic influx does not seem to differ between genitally normal mares and mares susceptible to endometritis (Ref 2.17). However in mares with PMIE, the neutrophils remain at high numbers, whereas they decline sharply in normal mares (Ref 2.11). Susceptibility to PMIE is not strongly correlated with endometrial biopsy grade, and chronic inflammatory cells, one of the determinants of endometrial category, are normal components of the equine endometrium and are not necessarily indicative of endometritis (Refs 2.18, 2.19). However endometrial biopsy remains a useful tool in determining degree of chronic irreversible change within the endometrium and collection of multiple biopsy samples do not affect fertility, even when they are collected at the same oestrus as breeding (Ref 2.20).

2.2 Chronic Uterine Infection

Clinical experience would show that mares that have PMIE at the start of the breeding season, can develop into mares with chronic uterine infection. Alternatively, mares with no previous history of PMIE can present with a uterine infection on initial examination. It is thought that in some of the mares with PMIE, defective uterine immune defence mechanisms may contribute to persistence of infection (Ref 2.15). It has clearly been shown that the uterine environment and defence mechanisms are strongly influenced by ovarian steroids, with progesterone having a suppressive effect (Refs 2.21, 2.22, 2.23, 2.24), although endometrial plasma cell numbers appear to be unaffected by hormone

treatment (Ref 2.25). However, mares prone to chronic uterine infection become infected after insemination in oestrus, even under the influence of oestrogen. Neutrophils entering the uterine lumen are the first line of immune defence against invading bacteria. Their migration from the blood is enhanced by chemotatic factors present in uterine fluid (Ref 2.26) which are increased after introduction of infection and inflammation into the uterus (Refs 2.14, 2.23 2.27). However a complex of equine neutrophil elastase and equine α-1 proteinase inhibitor, known to be chemotactic in other species, was not attractant for equine neutrophils (Ref 2.28). Uterine-derived neutrophils were not capable of migrating under agarose, possibly because neutrophils which have phagocytosed bacteria have run out of plasma membrane for locomotion (Ref 2.15). However, the importance of neutrophil migration once the cells have reached the site of inflammation is questionable. No defect in migration of blood-derived neutrophils has been found in susceptible mares (Ref 2.15).

Once neutrophils have reached the uterine lumen their capacity to ingest and kill bacteria is critical in elimination of infection. The phagocytic ability of uterine neutrophils from susceptible mares is less than that from resistant mares (Ref 2.15). It is likely that factors in uterine secretions from susceptible mares interfere with phagocytosis and uterine secretions from susceptible mares were significantly worse at promoting phagocytosis than secretions from resistant mares (Ref 2.15). Opsonisation by uterine secretions is dependent on both complement and specific antibody (Ref 2.29). Despite a widely held

belief that complement was deficient in chronically infected mares, haemolytic complement activity was high in uterine flushings from mares prone to chronic uterine infection, presumably because of persistent uterine inflammation (Ref 2.15). Although specific endometrial antibody titres to *Streptococcus zooepidemicus* were similar in resistant and susceptible mares, the opsonic activity of these antibodies was lower than those of resistant mares (Ref 2.30). The importance of antibody in elimination of uterine bacterial infection was further demonstrated by passive immunisation studies where hyper-immune serum was infused into the uterus of mares infected with the same strain of bacteria that the antiserum was raised against (Ref 2.31).

The cellular immune system has received little attention in the equine uterus. There appears to be no deficiency of T lymphocyte subsets $CD4^+$ or $CD8^+$ (Ref 2.19) in susceptible mares. Uterine MHC II expression was significantly upregulated in mares with endometritis, and a high level of immunostaining was seen in endometrial epithelial cells (Ref 2.18). Macrophage function seems to be normal in susceptible mares (Ref 2.32) although local antigen uptake by endometrial macrophages was not studied. However, we did show that macrophage numbers did not increase as much as would be expected in the endometrium of susceptible mares (Ref 2.33). This relative macrophage deficiency may be responsible for problems with antigen processing and handling at the uterine level.

2.3 Treatment

Treatment of ovariectomized mares with progesterone has been used as a model to induce chronic uterine infection in mares (Ref 2.34). Compared with oestrogen-treated and control mares, progesterone treatment decreased the opsonising capacity of uterine secretions (Ref 2.22), decreased the phagocytic activity of uterine neutrophils (Ref 2.21), decreased neutrophil migration *in vitro* (Ref 2.21), and altered the uterine environment (Ref 2.23). *S zooepidemicus* has the ability to adhere to endometrial epithelial cells *in vitro* (Ref 2.24) and progesterone treatment significantly increased bacterial adherence to epithelial cells. Oestradiol on the other hand upregulates expression of MHC Class II in the endometrium (Ref 2.18). Concentrations of endometrial oestrogen and progesterone receptors are also affected by the dominant circulating sex steroid hormone (Refs 2.35, 2.36). By using progesterone-treated mares as models for mares susceptible to endometritis, it was shown that transcervical manipulations had a profound effect on predisposing mares to uterine infections (Ref 2.37) and use of corticosteroid to reduce uterine inflammation in these mares was of little value (Ref 2.34). The adverse side-effects of using low volume infusion of irritant antibiotics has also been clearly demonstrated (Ref 2.38). Oxytocin administration to remove intrauterine fluid is a useful adjunct to other therapies. A combination of lavage and oxytocin therapy is now the preferred method of treatment. It is thought that antibiotics may not be necessary, even in cases of bacterial contamination, if mares are treated by large volume lavage and/or

ecbolic agents within 12 h of mating (Ref 2.10). Unless a specific bacterial species has been isolated in heavy pure culture, the indiscriminate administration of intrauterine antibiotics to all mares with PMIE cannot be justified and should be discouraged.

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ASSOCIATION OF UTERINE EDEMA WITH FOLLICLE WAVES AROUND THE ONSET OF THE BREEDING SEASON IN PONY MARES

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INTRODUCTION

In winter, most mares are anestrous and exhibit small hard ovaries with little follicular activity. In the transitional period between winter anestrus and normal cyclicity, mares often go through a period known as "shallow anestrus" or "spring transition". It is reported that during this time large follicles grow on the ovaries in approximately 50% of mares, and often reach preovulatory size, but fail to ovulate (1). An average of 3-4 waves of single large (<30 mm) follicles grow at intervals of approximately 10 days before one of these follicles finally goes on to ovulate (1,2). In the remaining mares the follicles fail to reach 35 mm in diameter and do not show a clear wave pattern. In spring transition, mares may remain in estrus for protracted periods of time. However their circulating concentrations of estradiol are low, because these large anovulatory follicles are steroidogenically incompetent (3,4). At this time, when the mares are showing estrus, it is difficult to deduce from transrectal ultrasound examination whether the large ovarian follicle will ovulate or regress. This can result in mares being mated repeatedly over a protracted period before the first ovulation in spring.

There is clearly a need for an accurate predictor of the ovulatory potential of a follicle prior to the onset of regular cyclicity. Concentrations of estrogen conjugates in urine have been shown to increase as first ovulation approaches (5), but these measurements are not readily available to veterinary practitioners or studfarms. In another study, mares appeared not to show estrous-like uterine edema more than 15 days before first ovulation in spring (6). Administration of estradiol to ovariectomized mares induces uterine edema (7) and it has been suggested that the presence of uterine edema is a

where the uterus appeared homogeneous with no anechoic areas. All of the authors were involved in ultrasonographic scanning the mares, but one of the authors (EDW) was present at the majority of the examinations and checked for consistency among operators. However in the results, "some uterine edema" was included with "no uterine edema", as it was likely that there were greatest individual operator differences in this category. Blood samples (20 mL) were collected from the jugular vein into evacuated heparinized tubes on the same days as the ovarian examinations. Plasma was separated by centrifugation at 2000 x g for 15 min at 4°C and stored at –20°C until assayed for estradiol concentrations. This sampling regime was maintained until the mares' second ovulation of the breeding season.

Estradiol assay

Concentrations of estradiol were measured in an extraction radioimmunoassay using a previously validated technique (9). The limit of detection of the assay was 2 pg/mL and the intra- and inter-assay coefficients of variation were 4.6 and 7.8%, respectively. Average recovery of estradiol from plasma was 91%.

Statistical analysis

Three different types of follicle waves were included: an anovulatory wave, the first ovulatory wave and second ovulatory wave. The anovulatory wave was chosen at random and was the first, second or third anovulatory wave. The effect of wave type on the number of days that uterine edema was present, the number of days that the large follicle (>25 mm) was present on the ovaries, and on estradiol concentrations (peak concentration measured in daily samples during the growth phase (>25 mm) of a follicle wave, and mean daily concentrations), and the proportion of time that

Anovulatory follicle waves (presence of a follicle > 25 mm) lasted an average of 16.3 days (range 5-27 days; Table 1). Uterine edema (Fig 1) was detected on at least one day in 7 of the 11 mares (64%). The uterine edema was recorded on an average of 2.8 days (range 0-8 days) during the anovulatory wave. Before first ovulation a large follicle (> 25 mm) was present for 12.4 days (range 7-19 days), and during this time edema was present for an average of 3.1 days (range 0-8 days). Twelve of the 14 mares (86%) had at least 1 day of uterine edema during follicular growth. By second ovulation a large follicle (> 25 mm) was present for a mean of 12.7 days (range 7-20 days). During this time edema was observed for an average of 4.5 days (range 2-8 days). All 11 mares (100%) showed uterine edema at some time during follicle growth.

Ten (91%) of the 11mares that had serial large anovulatory follicles, had uterine edema at some point prior to their first ovulatory estrus of the breeding season. The edema was first detected 16-86 days before first ovulation (mean = 43 $\pm$ 6.7 days before first ovulation). The 5 mares that did not have anovulatory follicle waves did not have uterine edema prior to first ovulatory estrus. A large follicle tended to be present longer during an anovulatory wave but this was not statistically different from the later waves because of the high variability in persistence of the anovulatory follicles. There was no significant difference among follicle wave types in the length of time that uterine edema was present (Table 1). However, uterine edema was present for a significantly greater proportion of time ($P < 0.05$) in the presence of a large follicle at first ovulation than during an anovulatory wave and was present relatively longest ($P<0.05$) at second ovulation (Table 1).

differences within mares would not be detected. Therefore it was considered to be valid in the present study to record and report only the presence or absence of estrous-like edema.

It is known that large transitional follicles are steroidogenically incompetent (3,4). In the present study, plasma estradiol concentrations were low in the presence of anovulatory follicles and only increased during growth of the first ovulatory follicle. Based on differences in uterine echogenicity, it has been suggested previously that estrogen concentrations may be lower at the first than at the second ovulation (6). However, we found no difference in peak or mean estradiol concentrations during growth of the preovulatory follicle between first and second ovulations, even though uterine edema was present for a proportionately longer period of time (days of uterine edema ÷ days follicle present) during growth of the second preovulatory follicle, than the first preovulatory follicle.

Although estrous behavior was not monitored in the present study, it is well recognised that transitional mares may remain in estrus for prolonged periods of time, despite low circulating concentrations of estradiol. We observed small elevations in estradiol concentrations during the growth of anovulatory follicle waves, and circulating concentrations were significantly higher on the days that uterine edema was present than on the days before and after detection of uterine edema. It seems likely therefore that in the absence of progesterone, the mare is highly sensitive to very low concentrations of estrogen during spring transition at the level of both the brain and the uterus. Indeed, a recent study has suggested that progesterone is responsible for dissipation of uterine edema in the cycling mare (7). Alternatively,

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Table 1. Uterine edema and plasma estradiol concentrations in mares with a large anovulatory or preovulatory ovarian follicle (>25 mm).

	Type of follicle wave		
	Anovulatory	1st ovulation	2nd ovulation
No of mares	11	14	11
Days follicle wave persisted > 25 mm	16.3 ± 2.5	12.4 ± 0.9	12.7 ± 1.3
Days uterine edema detected in the presence of a follicle > 25 mm	2.8 ± 0.9	3.1 ± 0.6	4.5 ± 0.6
Proportion (%) of time uterine edema detected in the presence of a follicle > 25 mm	17%[a]	25%[b]	35%[c]
Peak estradiol concentrations (pg/mL) in the presence of a follicular wave	9.0 ± 2.9[d]	30.1 ± 2.5[e]	29.4 ± 3.3[e]
Mean estradiol concentrations (pg/mL) in the presence of a follicular wave	5.9 ± 1.4[f]	17.1 ± 1.4[g]	16.6 ± 1.5[g]

Within a row, different superscripts represent a significant difference; a,b $P < 0.05$; a,c $P<0.01$; b,c $P < 0.05$; d,e $P<0.001$; f,g $P < 0.001$
All means are quoted ± SEM

WWF ewes where very few animals ovulate follicles from the penultimate wave of the cycle. In summary, the present results confirmed that a $PgF_{2\alpha}$/MAP regimen applied late in the oestrous cycle of ewes increased the ovulation rate, mainly through maintenance of ovulatory-sized follicles in the penultimate wave and their addition to ovulatory follicles from the final follicular wave before ovulation. Breakthrough ovulations that occurred during the period of MAP treatment were not associated with an increase in circulating concentrations of LH or FSH.

67. Development of highly steroidogenic non-apoptotic atreticic follicles in female Djungarian hamsters raised from birth under a short photoperiod.

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The aim of this study was to investigate the effects of a short day light regimen given from birth on ovarian follicular development and steroid hormone production in dwarf hamsters. Female Djungarian hamsters (*Phodopus sungorus*) were raised from birth in two different light regimes: i) 16h light : 8h dark (control photoperiod) and ii) 4h light : 20h dark (short photoperiod; SP). Hamsters were killed at 28, 56 and 80 days after birth (n=7/group) and blood and ovaries collected. One ovary was fixed in Bouin's solution, embedded in paraffin, cut in 5-µm sections and stained with haematoxylin and eosin, whereafter all healthy and atretic follicles were counted. The other ovary was frozen at –20 °C and the expression of 3β-hydroxy steroid dehydrogenase (3β-HSD) was enzyme-histochemically examined in 10-µm sections. Serum oestradiol-17β and progesterone concentrations were also determined. At all stages, the numbers of healthy preantral and antral follicles were lower ($p<0.05$) in SP than in control hamsters with antral follicles completely absent in 80-day-old SP animals. While the number of apoptotic antral follicles significantly decreased with age, an increasing number of non-apoptotic atretic follicles with a degenerated oocyte but intact non-proliferating, hypertrophied granulosa cells was observed in SP animals. These granulosa cells exhibit intensive 3β-HSD activity compared with interstitial cells, thecal cells, and granulosa cells of apototic follicles. Mean serum progesterone concentrations were similar in SP and control hamsters and varied from 2 to 6 pmol/l. Those of oestradiol varied from 132 to 542 and 325 to 2353 pmol/l in control and SP hamsters, respectively, with the highest concentrations present in 28-day-old SP animals. It is concluded that folliculogenesis is dramatically disturbed in Djungarian hamsters raised under SP. These animals develop numerous non-apoptotic atretic follicles with highly steroidogenic hypertrophied granulosa cells that are probably responsible for the increased serum oestradiol levels.

68. Expression of mRNA encoding P450scc, P450c17, and P450arom in equine follicles during spring transition and the breeding season.

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The period of spring transition between anoestrus and cyclicity is characterized by a resurgence of follicular activity, irregular exhibition of oestrous behaviour and resumption of secretion of gonadotrophins and ovarian steroids. During spring transition, large, steroidogenically incompetent follicles grow in the ovaries, and fail to ovulate. We hypothesized that these transitional follicles have defective steroidogenic enzyme expression. Ovaries were obtained from 14 pony mares on the day after detection of an actively growing 30 mm transitional anovulatory follicle, and also at the second oestrus of the breeding season on the day after the preovulatory follicle reached 30 mm. Dominant follicles were collected and snap frozen. The expression of mRNA encoding P450arom, P450scc, and P450c17 was measured by *in situ* hybridization. Differences in expression were analysed by split plot ANOVA. Size of dominant follicles at the time of removal was similar in transition (35 ± 0.2 mm) and at second oestrus (37 ± 0.2 mm). mRNA encoding P450arom was detected only in granulosa cells. There was a significantly lower level of mRNA encoding P450arom in transitional anovulatory follicles than in preovulatory follicles ($P<0.001$). mRNA encoding P450c17 and P450scc were detected only in theca cells. There were significantly lower levels of P450c17 and P450scc mRNA in the theca of transitional follicles than in preovulatory follicles ($P<0.001$). In conclusion, large equine follicles during spring transition have low levels of mRNA encoding steroidogenic enzymes which will contribute to the steroidogenic incompetence of dominant follicles during spring transition.

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69. Fetal ovarian development in inadequately grown porcine fetuses.

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Perturbed prenatal growth has a major impact on neonatal survival and recently it has been suggested that growth restriction during fetal life may affect reproductive performance in adult life. The naturally occurring within-litter variation in fetal size and the possibility to compare littermates, makes the pig an attractive model to investigate the repercussions of suboptimal growth in the development of reproductive organs. The present study compares the germ cell population in ovaries collected from the smallest fetus and a normally-grown littermate

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Research

Characterization of morphology and angiogenesis in follicles of mares during spring transition and the breeding season

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The mare is a seasonal breeder and undergoes a period of ovarian transition in spring between winter anoestrus and cyclicity. During spring transition LH concentrations are low and many mares have successive large anovulatory follicular waves which reach the size of preovulatory follicles. Follicular angiogenesis is essential for growth and health of preovulatory follicles. The aim of the present study was to investigate the morphology and vascularity of transitional anovulatory follicles. On gross inspection, the wall of transitional follicles was visibly less well vascularized than that of preovulatory follicles. Histologically, it could be seen that the theca was only poorly developed in transitional follicles. Immunostaining for factor VIII showed that there were significantly ($P < 0.05$) fewer blood vessels in the theca of transitional follicles. There was substantially less ($P < 0.001$) proliferative activity, measured by immunostaining for Ki67, in the endothelial cells and granulosa cells of transitional follicles compared with preovulatory follicles. Preovulatory follicles had a heavy band of immunostaining in the theca for vascular endothelial growth factor (VEGF), whereas staining was sparse in the transitional follicles. It was concluded that the poor vascularity and development of the theca layer in transitional follicles could be related to low circulating LH, and possibly other trophic hormones, and are likely to be the key factors in explaining the steroidogenic incompetence of transitional anovulatory follicles.

Introduction

The mare is a seasonal breeder and most mares enter a period of anoestrus during the short days of winter. During this time only very small follicles are present in the ovaries. In many mares there is a transitional period between deep winter anoestrus and normal ovarian cyclicity, when larger follicles grow and regress. In approximately 50% of mares, sequential anovulatory follicular waves then develop, with the dominant follicle reaching a diameter similar to that of a preovulatory follicle (Ginther, 1990). These follicles fail to ovulate because of suppression of GnRH secretion by inhibitory neuronal mechanisms that results in lack of LH stimulation (Fitzgerald and Mellbye, 1988; Aurich *et al.*, 2000). Furthermore, these large transitional follicles are thought to be steroidogenically incompetent as circulating oestradiol concentrations are very low, and incubated granulosa cells from anovulatory transitional follicles produce significantly lower concentrations of oestradiol than do granulosa cells from preovulatory follicles (Davis and Sharp, 1991).

As daylength increases, an increase in GnRH secretion results in FSH secretion and follicle growth (Freedman *et al.*, 1979; Turner *et al.*, 1979). LH secretion does not increase as pituitary reserves are low in winter (Silvia *et al.*, 1987). However, shortly before first ovulation there is a large increase in circulating LH which coincides temporally with an increase in plasma oestrogen concentrations. It is thought that the increase in circulating oestradiol is the key event in increasing LH synthesis and secretion at the pituitary, or in increasing GnRH release (Sharp *et al.*, 1991). The reason for the apparently low steroidogenic capacity of transitional follicles is unclear. There is significant *in vitro* conversion of labelled androstenedione to oestrogens by transitional follicles (Seamans and Sharp, 1982) and so it appears that these follicles have sufficient quantities of the aromatizing enzyme, $P450_{arom}$. There is circumstantial evidence that the rate-limiting step in oestrogen synthesis in transitional follicles may be insufficient amounts of androgen substrate caused by low concentrations of P450C17. Concentrations of progesterone were similar in large follicles throughout transition and into cyclicity, but concentrations of androgen did not increase until the onset of cyclicity (Davis and Sharp, 1991). Although there may be deficiencies in steroidogenic enzymes in transitional follicles, an alternative explanation may be that the development of the transitional follicles is not as advanced as that of preovulatory follicles, and they may be less well endowed with blood vessels, thus limiting availability of substrate for steroidogenesis. Indeed, there is anecdotal evidence that, on gross inspection, transitional follicles appear to be relatively avascular (Sharp and Davis, 1993).

The regulatory role of gonadotrophins in follicle development has been well documented; however, the mechanisms acting locally within the follicle that translate hormonal stimulation into growth and differentiation are

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not well understood. Blood vessel development has a crucial role in follicular maturation (Richards, 1980) and, during follicle growth, a rich capillary plexus develops in the thecal layer surrounding the avascular granulosa cells. Studies in primates have shown that the density of the microvascular network of follicles destined to ovulate is at least double that of follicles destined to become atretic (Zeleznik *et al.*, 1981). This increased vascularity results in increased delivery of gonadotrophins to preovulatory follicles. Ravindranath *et al.* (1992) showed that there is an apparent association between the capacity of a follicle to produce angiogenic factors and follicle selection. Angiogenesis requires proliferation and migration of vascular endothelial cells. Several intraovarian regulators have been implicated in angiogenesis. Vascular endothelial growth factor (VEGF) is a multifunctional cytokine that stimulates blood vessel formation and enhances microvascular permeability with 50 000 times the potency of histamine (Dvorak *et al.*, 1995). Expression of VEGF mRNA and protein has been reported in follicles and corpora lutea of various species (Ravindranath *et al.*, 1992; Gordon *et al.*, 1996; Yamamoto *et al.*, 1997; Barboni *et al.*, 2000; Toutges *et al.*, 2000; Kashida *et al.*, 2001). There appears to be a clear difference in patterns of expression among species.

Very little is known about the relative vascularity of equine ovulatory and anovulatory transitional follicles, although it is clear that the degree of vascularization may be a critical factor in determining their subsequent fate. Therefore, in the present study we used immunohistochemistry to detect the presence of VEGF in transitional and preovulatory follicles. The proliferative activity in granulosa cells and endothelial cells was investigated by determining expression of the Ki67 antigen, and the distribution and neoformation of blood vessels was confirmed by immunolocalization of the endothelial cell marker, Von Willebrand factor VIII.

Materials and Methods

Six Pony mares of mixed breeding, weighing 344–445 kg and aged 3–19 years, were used. The reproductive history of the mares was unknown, but all were in winter anoestrus at the beginning of the period of study. The ovaries of the mares were examined three times a week by transrectal ultrasonography from the beginning of February until the onset of follicular waves. During one of the follicular waves, the ovary containing the dominant follicle was removed by a colpotomy incision after appropriate sedation and analgesia (Watson and Sertich, 1990) on the day after the dominant follicle reached 30 mm in diameter. The mares were then monitored by transrectal ultrasonography until first ovulation, which occurred between 13 April and 10 May. The remaining ovary was removed at either the second or third subsequent oestrus on the day after the dominant follicle reached 30 mm in diameter. Care was taken to remove ovaries only when the diameter of the follicle was increasing each day.

Immediately after ovariectomy, excized ovaries were transported on ice to the laboratory for dissection. After identification of the largest follicle, the tunica albuginea was removed carefully with fine scissors and forceps, and the follicle diameter was measured and recorded. A syringe and fine gauge needle were used to aspirate follicular fluid, which was stored at –20°C until assayed for oestradiol. The follicle was re-inflated with air and the anterior follicle wall was cut open. The appearance of the follicle wall was recorded. One piece of follicle wall, approximately 0.5 cm × 0.5 cm, was immersed in freshly prepared 4% (w/v) paraformaldehyde. The tissue was kept at 4°C and transferred 24 h later to 70% (v/v) alcohol until it was embedded in paraffin wax.

Immunostaining

Immunostaining was performed using the following antibodies: rabbit anti-VEGF (Santa Cruz Biotechnology, Santa Cruz, CA), rabbit anti human Von Willebrand factor VIII (Dako Laboratories, High Wycombe) and a monoclonal antibody directed against the nuclear non histone antigen, Ki67 (Novocastra, Peterborough). Paraffin wax sections (4 μm thickness) were mounted on slides coated with BioBond (British Biocell Int., Cardiff), deparaffinized and rehydrated. The sections were treated with 0.3% (v/v) hydrogen peroxide and incubated for 30 min with 35% goat serum to block non-specific binding. The sections were incubated with primary antibody or 2% normal rabbit serum (VEGF and factor VIII) or 2% normal mouse serum (Ki67). Dilution and incubation times and temperatures for the different antibodies were: 1:200 for 120 min at room temperature for VEGF; 1:250 for 90 min at room temperature for factor VIII; and 1:40 for 3 h at 37°C for Ki67. The slides were subsequently incubated for 30 min with biotinylated goat anti rabbit immunoglobulins (Vector Laboratories, Peterborough) or goat anti mouse immunoglobulins (Vector Laboratories) for Ki67, diluted to 1:100 in phosphate buffered saline (PBS), or to 1:200 for Ki67. The slides were washed in PBS, treated with avidin–peroxidase complex (Vector Laboratories) and stained with 0.05% (w/v) 3,3'-diaminobenzidine containing 0.01% (v/v) hydrogen peroxide. Counterstaining was performed with Meyer's haematoxylin and the sections were rinsed with Scott's tap water.

Area of Von Willebrand factor VIII immunostaining

The Quantimet image processing and analysis system 500 (Leica, Cambridge) was used to measure the area of immunostaining for factor VIII. Regions from the inner, middle and outer areas of the sections were randomly selected. Two sections and three fields per section were counted. The system was optimized for each individual section based on the density of the stain. The area used in the analysis was calibrated at × 200 magnification. The area of factor VIII immunostaining for each group was expressed as mean ± SEM per unit area.

Labelling index for cell proliferation

Sections were examined at × 400 magnification. The number of nuclei positively stained for Ki67 and the total number of nuclei were counted in two sections per animal and four fields per section, and the percentage of positively stained nuclei (label index) was calculated.

Hormone assays

Concentrations of progesterone and oestradiol were measured directly in follicular fluid using assays described previously (Corrie *et al.*, 1981; Glasier *et al.*, 1989; Law *et al.*, 1992; Watson *et al.*, 2000; Riley *et al.*, 2001). Assay sensitivity was 0.5 ng ml^{-1} for progesterone and 8 pg ml^{-1} for oestradiol. Intra- and interassay coefficients of variation were 9.0 and 12.6%, respectively, for progesterone, and 4.6 and 7.8%, respectively, for oestradiol. Displacement curves produced by serial dilutions of follicular fluid and curves produced by addition of hormone to follicular fluid samples were parallel to the respective standard curves.

Statistical analyses

Hormone concentrations in follicular fluid from transitional and preovulatory follicles from the same mares, as well as follicle size, were compared by paired *t* test. Hormone data were log-transformed before analysis. The area of staining for factor VIII and the proportion of cells staining positively for Ki67 in transitional and preovulatory follicles were compared using a paired *t* test.

Results

Size of follicles at removal was not significantly different between the transitional (33.8 ± 2.3 mm) and preovulatory (34.1 ± 1.5 mm) groups. The colour of the walls of the transitional follicles was yellowish to pale pink, and that of the preovulatory follicles was orange to red. The concentration of oestradiol in follicular fluid was significantly lower ($P < 0.01$) in the transitional follicles (345 ± 112.3 ng ml^{-1}) than in the preovulatory follicles (1063 ± 169.2 ng ml^{-1}). Similarly, progesterone concentrations were significantly lower ($P < 0.05$) in transitional follicles (6.2 ± 1.7 ng ml^{-1}) than in preovulatory follicles (33.6 ± 12.8 ng ml^{-1}).

Histological findings

Both anovulatory and preovulatory follicles had a well-organized layer of granulosa cells in contact with the basement membrane (Fig. 1). The granulosa cells appeared to be randomly distributed in the upper layers. There did not appear to be any difference in the thickness of the granulosa layers in anovulatory or preovulatory follicles, although there was some variability between follicles. Occasional mitotic nuclei were visible in both transitional and preovulatory follicles, and a few pycnotic nuclei were observed. The theca interna in preovulatory follicles comprised a thick layer of plump polyhedral cells with a pale nucleus and cytoplasm (Fig. 1a), whereas the theca layer of the anovulatory follicles was thin and poorly developed in most parts with only sparse foci of polyhedral cells (Fig. 1b). Most of the theca cells retained a fibroblast-type appearance with a spindle-shaped darkly staining nucleus.

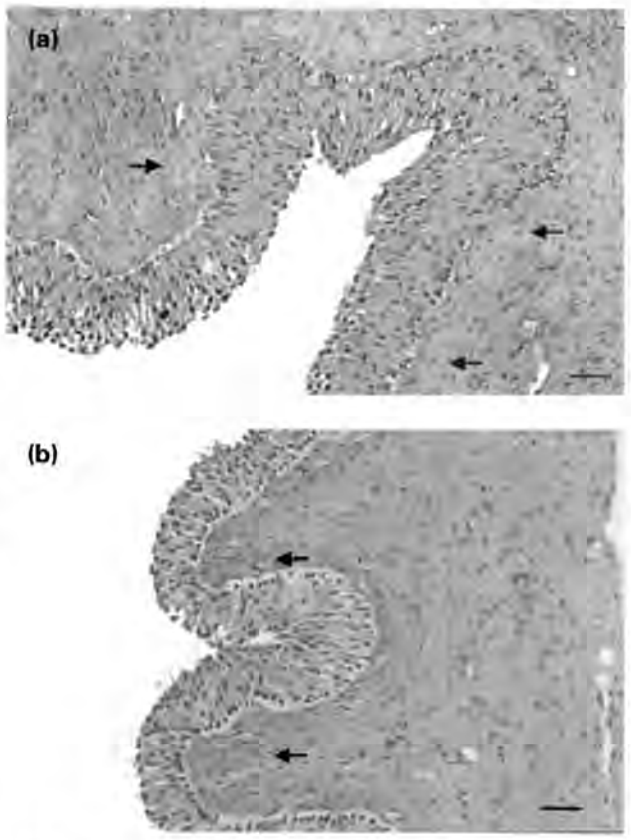

Fig. 1. Morphology of follicular walls from (a) a preovulatory and (b) a transitional anovulatory equine follicle. The theca interna in the transitional follicle is thin and lacks the plump polyhedral cells visible in the preovulatory follicle (arrows). Sections stained with haematoxylin and eosin. Scale bars represent 50 μm.

Immunohistochemical localization of VEGF, factor VIII and Ki67

Immunostaining for VEGF was confined mainly to the theca interna. The granulosa layer remained mainly unstained, with a few scattered positively stained cells. In preovulatory follicles, the entire theca interna layer stained strongly and diffusely for VEGF (Fig. 2a). Immunostaining in the transitional follicles was scant, with patchy staining in the thin theca layer (Fig. 2b).

Immunostaining for factor VIII was confined to endothelial cells of blood vessels in the theca interna. The theca was well supplied with blood vessels in the preovulatory follicles, whereas the transitional follicles had a relatively avascular theca. A significantly larger ($P < 0.05$) area of

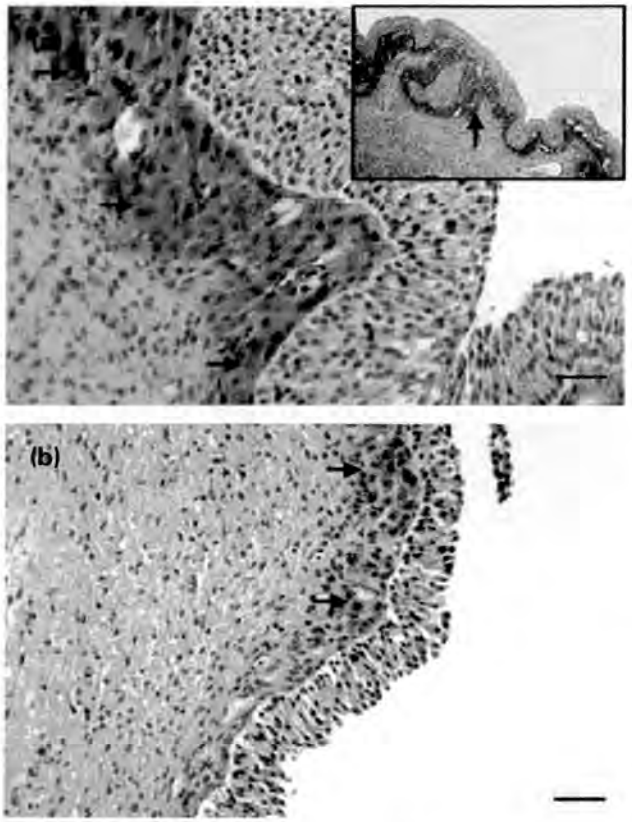

Fig. 2. Immunohistochemical staining for vascular endothelial growth factor (VEGF) in (a) a preovulatory follicle and (b) a transitional anovulatory equine follicle. Note the broad band of staining in the theca of the preovulatory follicle compared with the sparse staining in the transitional follicle (arrows). Inset shows low magnification of preovulatory follicle with intense staining in theca. Scale bars represent 50 μm.

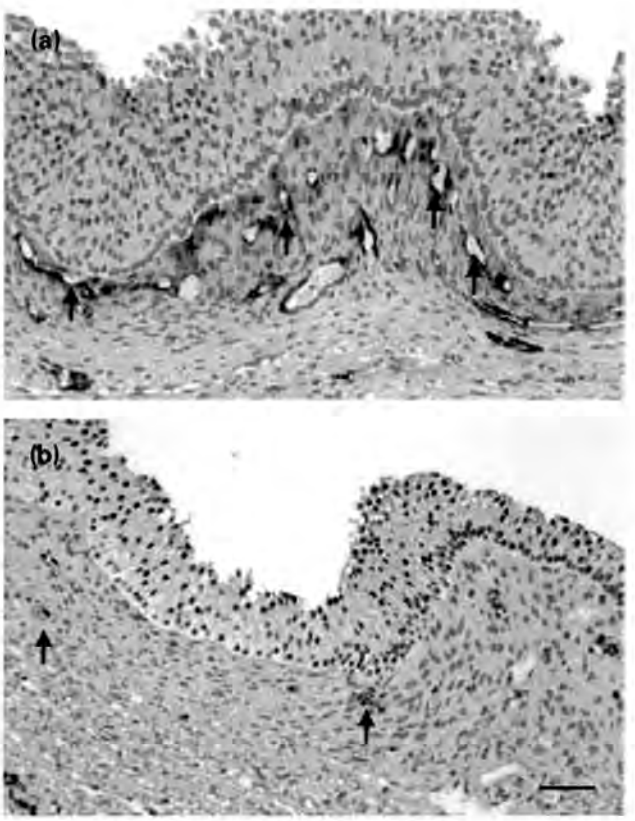

Fig. 3. Immunohistochemical staining for factor VIII in (a) a preovulatory follicle and (b) a transitional anovulatory equine follicle. Staining is confined to the theca (arrows). Scale bars represent 50 μm.

tissue was stained for factor VIII in the preovulatory follicles than in the transitional follicles (Figs 3 and 4).

Positive staining for Ki67 was confined to cell nuclei and was frequently present in the granulosa cells and thecal endothelial cells of preovulatory follicles (Fig. 5a). No staining was visible in endothelial cells of transitional follicles and only occasional granulosa cells were stained (Fig. 5b). The difference in labelling index for Ki67 between preovulatory and transitional follicles was highly significant (Fig. 1).

Observations for immunohistochemical staining were consistent across all six animals studied. No positive immunostaining was observed in negative control sections (Fig. 6).

Discussion

The results of the present study show for the first time clear differences in morphology and vascularization between large follicles collected during spring transition (anovulatory) and the breeding season (preovulatory) from mares. The major differences reported in this study, together with previously reported differences in circulating gonadotrophin concentrations, could play a key role in determining the subsequent fate of these two types of follicle.

A follicle diameter of approximately 35 mm was chosen because it is known that equine preovulatory follicles are responsive to exogenous gonadotrophin stimulation at this size and ovulate within 48 h (Duchamp *et al.*, 1987). All of the follicles collected in the present study were still growing and were not regressing; this was confirmed by histological evaluation of granulosa and theca cell morphology. The morphological differences in the theca interna layer of the preovulatory and transitional follicles were marked. The preovulatory follicles had the characteristic plump polyhedral cells that have been described previously (Kenney *et al.*, 1979; Kerban *et al.*, 1999). It has been suggested that these cells have undergone luteinization (Kenney *et al.*, 1979) and it is known that follicular progesterone concentrations increase during the preovulatory period in mares (Watson and Sertich, 1990). Furthermore, follicular progesterone concentrations were increased in the preovulatory follicles sampled in the present study. It has also been suggested that a thick theca layer may be essential in the growing pre-

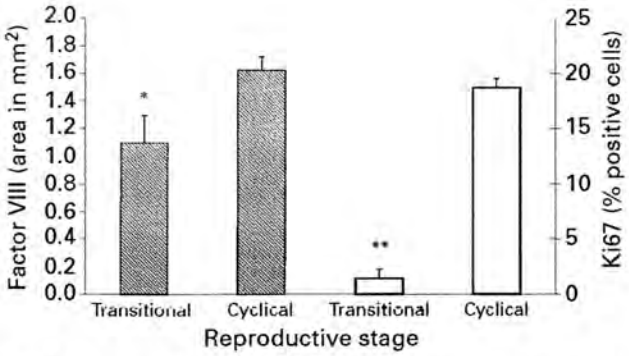

Fig. 4. Area of staining for factor VIII (▨) and percentage of cells positive for Ki67 (□) in transitional and cyclical preovulatory follicles from six mares. $^{*}P < 0.05$ and $^{**}P < 0.001$ compared with cyclical value.

ovulatory follicle for provision of androgen substrate to maintain follicular oestradiol synthesis. Therefore, it seems likely that, as this layer is only poorly developed in transitional follicles, the lack of androgen substrate will contribute substantially to the low oestradiol concentrations measured in these follicles. Hence, the morphological findings of the present study explain the low steroidogenic capacity of incubated walls of transitional follicles (Davis and Sharp, 1991) and indicate strongly that the deficiency in thecal P450C17 suggested by Davis and Sharp (1991) is directly attributable to poor thecal development.

Poor vascularity, characterized by visibly pale follicular walls, has been reported to be a sign of atresia in equine follicles (Kenney *et al.*, 1979; Mlodawska and Okolski, 1997; Pedersen, 2000). In the present study, the lining of transitional follicles was paler than that of preovulatory follicles. This observation was confirmed by immunostaining for factor VIII, which identifies endothelial cells. There was a significantly smaller area of factor VIII immunostaining in the theca of transitional follicles compared with preovulatory follicles; this does not necessarily mean that there was a greater number of individual blood vessels, as the same vessel may intersect the plane of the section any number of times. However, the results of the present study indicate strongly that there is increased vascularity in the preovulatory follicles compared with transitional follicles as a result of increased numbers of vessels, increased size of the vessels or increased tortuosity of the vessels, or a combination of these factors. Furthermore, the transitional follicles contained very little VEGF, which is important in promoting angiogenesis, whereas VEGF appeared to be abundant in the preovulatory follicles. VEGF also has the ability to increase the permeability of the microvasculature (Murohara *et al.*, 1998). Thus, in preovulatory follicles, the richer blood supply in combination with increased permeability of blood vessels will allow increased provision of oxygen, nutrients and substrates, as well as circulating gonadotrophins, which are essential for follicular health, growth and steroidogenesis. In addition, the rich blood supply to preovulatory follicles will allow more follicular steroids and other hormones to leave the follicular fluid and access the circulation.

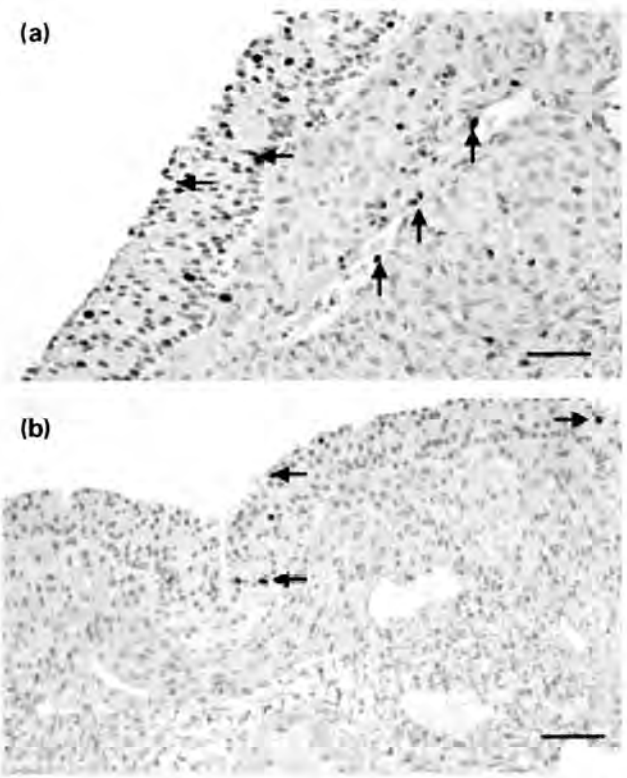

Fig. 5. Immunohistochemical staining for Ki67 in (a) a preovulatory follicle and (b) a transitional equine follicle. Note positive nuclear staining in granulosa cells (horizontal arrows) and in endothelial cells in the preovulatory follicles only (vertical arrows). Scale bars represent 40 μm.

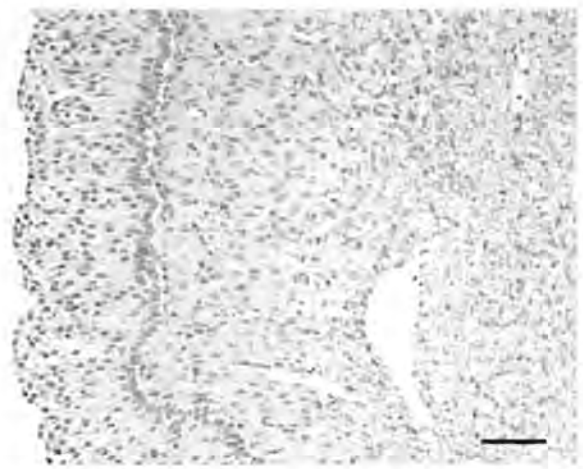

Fig. 6. Negative control section of an equine preovulatory follicle stained for Ki67 in which the primary antibody was substituted with 2% normal rabbit serum. Negative control sections for the other antigens similarly lacked positive staining. Scale bar represents 50 μm.

The VEGF protein was clearly present in the thecal cells of equine follicles, which is similar to human follicles (Gordon *et al.*, 1996; Yamamoto *et al.*, 1997). VEGF protein and mRNA encoding VEGF have been identified in both granulosa and theca cells from follicles of cows (Berisha *et al.*, 2000) and pigs (Barboni *et al.*, 2000). Isolated granulosa cells from various species secrete VEGF in culture (Christenson and Stouffer, 1997; Hazzard *et al.*, 1999; Barboni *et al.*, 2000). These differences may in part reflect species differences or disparity in stages of follicle maturity at the time of collection, but may also indicate that VEGF is being produced in the granulosa cells and that the protein then passes to the theca layer where the blood vessels are located. Post transcriptional regulation of VEGF production by gonadotrophins may take place in periovulatory follicles (Hazzard *et al.*, 1999).

The growth of new blood vessels can also be monitored by measuring proliferation of endothelial cells (Jablonka-Shariff *et al.*, 1993; Rodger *et al.*, 1997). Immunohistochemical detection of the proliferation marker Ki67 has been used previously as a monitor of mitotic activity in equine endometrium (Gerstenberg *et al.*, 1999). Cells with the morphology of endothelial cells that stained positively for Ki67 were present only in the theca of preovulatory follicles, which is indicative of active proliferation of blood vessels in these follicles. Many of the granulosa cells in these follicles were also positively stained, whereas staining was infrequent in the granulosa of the transitional follicles, indicating active cell division in the preovulatory follicles in contrast to the transitional follicles. In the marmoset corpus luteum, it appears that luteal cells that stain positively for Ki67 do not co-label with the steroidogenic cell marker 3β-HSD (Young *et al.*, 2000), indicating that steroidogenic cells may not produce steroid hormones and express cell cycle antigens simultaneously. It is not known whether this is also true in the follicle, but the preovulatory follicles in the present study with high proliferation indices were markedly more steroidogenically active than the transitional follicles. It has been shown that activation of VEGF production is more dependent on the dynamic status of the follicle and its growth rate than on its diameter (Barboni *et al.*, 2000). This finding correlates well with the high degree of proliferative activity in the preovulatory follicles in the present study.

The low vascularization in the transitional follicles in the present study, together with the low proliferative index, low VEGF protein content and follicular steroids are consistent with early follicular atresia, although this was not evident histologically. As seen in the present study in equine follicles, endothelial cell proliferation has been strongly correlated with expression of VEGF in theca cells during follicle growth (Yamamoto *et al.*, 1997). Follicle health depends on the presence of a rich network of capillaries in the innermost part of the thecal wall, such that oxygen and nutrients can be transported into the follicle, particularly at the cumulus–oocyte complex. Early signs of atresia are characterized by disappearance of this inner vascular layer (Hay *et al.*, 1976). Hypoxia is commonly implicated in stimulation of VEGF in most tissues, but in ovarian cells, a relationship has been shown between gonadotrophin stimulation of cultured luteinized granulosa cells and VEGF production (Christenson and Stouffer, 1997; Hazzard *et al.*, 1999). Other studies have shown *in vivo* that the intense angiogenesis in the early primate corpus luteum is dependent on gonadotrophin stimulation of the luteal cells (Dickson and Fraser, 2000) and VEGF concentrations in follicular fluid from prepubertal gilts increase after eCG and hCG treatment (Barboni *et al.*, 2000). As follicle health and growth are also dependent on gonadotrophin stimulation, it is likely that the low circulating concentrations of LH, characteristic of the transitional breeding season (Freedman *et al.*, 1979), failed to stimulate adequate production of angiogenic factors, including VEGF. Other trophic hormones such as growth hormone, which is known to be low in mares during seasonal anoestrus (Aurich *et al.*, 1999) and is involved in follicle growth and viability (Kirkwood *et al.*, 1990), may also be involved. The low concentrations of angiogenic factors then led to poor thecal vascularization and vascular permeability, both of which are essential for trophic support of the actively dividing follicular cells. In turn, poor vascularity will contribute to inadequate delivery of gonadotrophins and other trophic factors to the follicle to sustain development. Therefore, in these mares, the low concentrations of thecal VEGF could result in failure of further development and subsequent atresia of transitional follicles. In primates, VEGF production has been implicated in follicle selection during the menstrual cycle (Ravindranath *et al.*, 1992). From the results of the present study it appears likely that VEGF could also be a key factor in regulating gonadotrophin dependent follicular growth in equine preovulatory follicles.

It is possible to draw three main conclusions on the differences between transitional and preovulatory follicles: (i) the histological morphology of these two types of follicle is clearly different: the thecal layer is only poorly developed in transitional follicles and the proliferative index of the granulosa cells is markedly depressed in transitional follicles; (ii) thecal vascularization is sparse in transitional follicles in association with the absence of proliferative activity in the vascular endothelial cells; and (iii) VEGF, one of the main angiogenic factors, is present in abundance in the theca of preovulatory, but not transitional, follicles. These inter-related factors could explain the low steroidogenic capacity of transitional follicles and why these follicles fail to progress to ovulation during the spring anovulatory period in mares.

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(1) support primordial follicle growth and (2) investigate the mechanisms regulating early follicle growth. This study aimed to quantify follicle density and viability *in situ* in ovarian cortex, without compromising follicle developmental potential *in vitro*. Ovine cortical tissue was cut into 5mm^2 pieces and cryopreserved. After thawing, tissue was sectioned to 80-100μm thickness. Cortical slices (n=346) were incubated in 50μg/ml neutral red dye (NR) for 4 hrs and the number, diameter and morphology of stained structures were recorded before and after isolation. Additionally, NR positive (n=49) and negative (n=118) slices were fixed for evaluation by haematoxylin and eosin staining, or TUNEL labelling to assess follicle health status. NR positive (n=52) and negative (n=11) slices were also cultured in serum-free media on Millicell inserts for 8 days at 37°C, 5% CO_2. Following culture, viability was confirmed in 42 NR positive follicles using 5(and 6) carboxyfluorescein diacetate succinimidyl ester (CFDA-SE). The results showed that NR dye concentrated in follicular structures within weakly stained stromal tissue in 41.7% (n=147) of tissue slices. Slices that were NR negative only contained follicles with high levels of follicular apoptosis (74.1% granulosa cell, 75% oocyte TUNEL positive). In contrast, fewer TUNEL staining follicles were present in NR positive slices, with lower levels of apoptosis (49.2% granulosa cell, 60.1% oocyte) suggesting preferential detection of healthy follicles by NR. After culture, 61.5% of positive slices re-stained with NR and 92.8% of the isolated follicles were viable by CFDA-SE staining. This study shows that NR dye can be used to predict viable follicle density *in situ* in slices of cryopreserved ovarian cortex. Incubation in NR prior to culture does not compromise subsequent follicle development *in vitro*.

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35. Effects of activin A on embryo development during implantation *in vitro*.

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Activin A, a member of the inhibin family of growth factors, has been shown to influence various aspects of mammalian cell development including proliferation, death and differentiation. Since activin A has also been shown to influence oocyte maturation and mesoderm induction our aim was to determine whether it has similar roles during implantation using an *in vitro* model. Mouse embryos (MF-1) were cultured from the 2-cell stage in either the absence (A0) or presence of recombinant human activin A at 10ng/ml (A10) or 100ng/ml (A100). On day.6 hatched blastocysts (n=60 per treatment group over 3 experiments) were transferred to fibronectin-coated coverslips in the same but fresh medium. On day 9 attached embryos were stained using TUNEL to determine apoptotic and total cell numbers. Embryos were scored for the presence of trophoblast outgrowths (-, +, ++ or +++). The surface area, length and width of each embryo were quantified using image analysis (Scion software). Nuclear phenotype was also assessed as a possible indicator of differentiation. Data analysis was performed using ANOVA. Embryos cultured in the presence of activin A had significantly higher total cell numbers (A0: 98.8 ± 4.9, A10: 105.8 ± 4.5, A100: 122.9 ± 5.6; $p<0.005$) and lower dead cell indices (apoptotic/total) (A0: 0.188 ± 0.015, A10: 0.150 ± 0.011, A100: 0.098 ± 0.008; $p<0.001$) compared to the control. Activin A also significantly inhibited trophoblast outgrowth in a concentration dependent manner as assessed using the semi-quantitative system ($p<0.001$, X^2). Moreover, the surface area/total cell number (A0: 225.6 ± 23.0, A10: 193.9 ± 11.4, A100: 151.5 ± 6.6; $p<0.01$) and length/width ratios were significantly lower (A0: 0.562 ± 0.023, A10: 0.641 ± 0.025, A100: 0.689 ± 0.021; $p<0.001$). In addition, the presence of activin A significantly reduced the proportion of cells with a large, nuclear phenotype (A0: 10.0%, A10: 5.3%, A100: 3.5%; $p<0.001$). In conclusion, the data suggests that activin A may influence cell proliferation and death, as well as trophoblast outgrowth and differentiation during mouse blastocyst implantation.

36. The effect of $PGF_{2\alpha}$ on persistent corpus luteum in Sahiwal cows.

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In dairy animals, there are so many different conditions, which affect reproductive performance, and persistent corpus luteum (PCL) is one of those. It was suggested that this condition was difficult to treat and consequently result in lengthening of service period and calving interval. If this condition was not diagnosed and treated timely may result into permanent infertility and thereby huge loss to the breeders. The commercial availability of $PGF_{2\alpha}$ and its analogues had promoted management and treatment of PCL in other part of the world on different breeds of cows with variable rates of success. This study was designed to determine the response of treatment of Sahiwal cows identified as having PCL with $PGF_{2\alpha}$ (Dalmazin: Fatro Pharmaceutical). The present investigation has been conducted on Sahiwal cows maintained at Livestock Experimental Station, Bahadurnagar, Okara (Pakistan). The cows after 80-90 days post-partum were examined per rectum to check the condition of genitalia for PCL and this was also confirmed by progesterone assay in weekly blood samples for three weeks. All cows (n=20: mean weight, 402.0 ± 6.1kg) suffering from PCL was treated with 2 ml of Dalmazin intramuscularly on the same day. It was found that 85% of animals as determined by the examination of reproductive tracts and confirmed by progesterone analysis became normal and consequently 60% were pregnant at first service whereas 25% were repeat breeder. In conclusion, the treatment of Sahiwal cows, which are suffering from PCL, with $PGF_{2\alpha}$ may correct this condition successfully.

37. Concentrations of inhibin and steroids in follicles from mares during spring transition and the breeding season.

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In the period between winter anoestrus and cyclicity, many mares exhibit a period of transitional ovarian activity when there is cyclical growth and regression of large follicles in the absence of ovulation. Dominant anovulatory and preovulatory follicles

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Abstract

Insulin-like growth factor binding protein-2 (IGFBP-2) mRNA in equine follicles during vernal transition and the breeding season

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1. Introduction

The period of vernal transition between anestrus and cyclicity is characterized by a resurgence of follicular activity, irregular exhibition of estrous behavior and resumption of secretion of gonadotropins and ovarian steroids. In many mares, during vernal transition, large dominant follicles grow and regress, but do not ovulate [1]. These follicles appear to be steroidogenically incompetent. It is known that the insulin-like factor (IGF) system, which is composed of IGF-I and -II, IGF receptors, IGF-binding proteins (IGFBPs), and IGFBP proteases play an essential role in modulating the response of follicles to gonadotropins. The IGFs stimulate granulosa cell proliferation and interact with FSH to control estradiol production by granulosa cells [2]. IGFBPs are involved in regulating this process by virtue of their ability to bind IGFs with high affinity and so regulate IGF bioavailability. Changes in the levels of IGFBPs in follicular fluid correlate with functional changes in the ovarian follicles. In many species, follicular growth and atresia are characterized by dramatic changes in intrafollicular IGFBP concentrations, reflecting changes in expression and in intrafollicular proteolytic activity which degrades IGFBP-2, -4, and -5. A recent study has identified IGFBPs in equine serum and follicular fluid and has shown that they are clearly related to the steroidogenic capacity and physiological status of equine follicles [3]. IGFBP-2 has been postulated to be involved in regulating the bioavailability of IGF during the final stages of follicle growth in ruminants [4]. We aim to test the hypothesis that the expression of IGFBP-2 in large (>30 mm) follicles of mares during vernal transition will be higher than in cycling mares. We propose that this would effectively reduce the bioavailability of intrafollicular IGF during vernal transition

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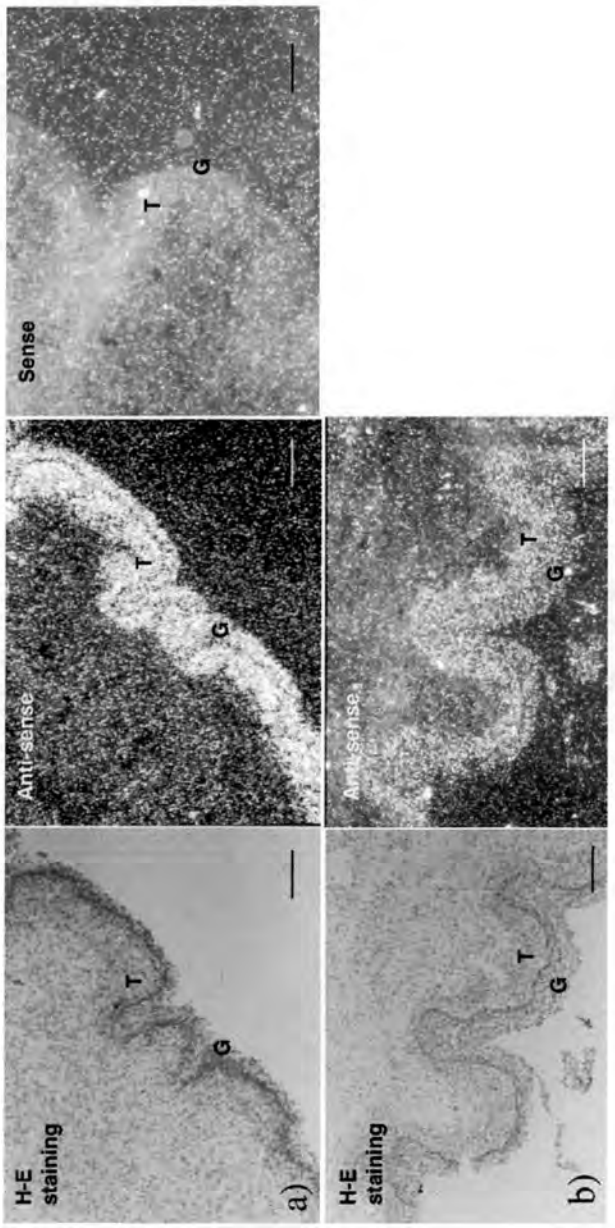

Fig. 1. In situ hybridization showing IGFBP-2 mRNA expression in granulosa and theca cells in a dominant estrous follicle (a) and in a transitional follicle (b). Scale bar represents 100 μm.

thus reducing sensitivity to FSH when compared to ovulatory follicles during the breeding season and, at least in part, explain steroidogenic incompetence during transition. The specific aims of this study were, therefore, to describe the spatial pattern of expression of mRNA encoding IGFBP-2 in large equine follicles and to compare the level of expression in dominant follicles collected during vernal transition and the breeding season.

2. Materials and methods

The ovaries of 14 mares (240–450 kg, aged 3–20 years) were scanned daily from mid-January throughout vernal transition. Ovariectomies were performed on the day after detection of an actively growing 30 mm transitional follicle, and also at the second estrus of the breeding season on the day after the preovulatory follicle reached 30 mm. A sample of follicular fluid was frozen at −20 °C for assay of estradiol concentrations. Dominant follicle walls were collected and snap frozen. Frozen sections (14 μm) were cut and the expression of mRNA encoding IGFBP-2 was measured by in situ hybridization using an ^{35}S-labeled bovine IGFBP-2 riboprobe. After a series of high stringency washes, the expression (expressed as the number of pixels within a defined area of the tissue occupied by a silver grain) was quantified by image analysis. Differences in expression between follicles obtained during the vernal transition and breeding season were analyzed by split plot ANOVA using GenStat Release 4.21.

3. Results

Size of dominant follicles at the time of removal was similar in transition (34.7 ± 0.21 mm) and at second estrus (36.6 ± 0.21 mm). Dominant follicles collected at the second estrus contained significantly higher ($P < 0.05$) concentrations of estradiol (1000 ± 124.6 ng/ml) than the anovulatory spring follicles (353 ± 131 ng/ml). The mRNA encoding IGFBP-2 was detected in granulosa and theca tissue of large follicles obtained from mares during vernal transition and the breeding season (Fig. 1). In addition, high expression was associated with a subset of blood vessels in stromal tissues (Fig. 2). There

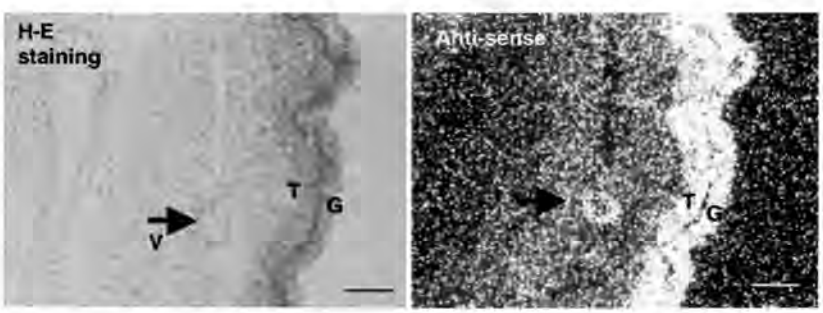

Fig. 2. In situ hybridization showing IGFBP-2 expression in vasculature (→) in stroma in a dominant estrous follicle. Scale bar represents 100 μm. T: theca cells; G: granulosa cells; V: blood vessel.

was a significantly lower level ($P < 0.001$) of IGFBP-2 mRNA expression in theca cells of transitional follicles than in estrous follicles (11.0 and 18.9, respectively, S.E.D. = 1.60; $n = 14$). In contrast, there was no significant difference ($P = 0.71$) in IGFBP-2 mRNA expression in granulosa cells between transitional and estrous follicles (12.7 and 13.2 respectively, S.E.D. = 1.32; $n = 14$).

4. Discussion

The results indicate that mRNA encoding IGFBP-2 is expressed in granulosa and theca from large (>30 mm) equine follicles during vernal transition and the breeding season. Expression was significantly higher in thecal tissue collected during the breeding season compared to vernal transition. In contrast there was no difference in expression in granulosa cells between these two stages. The spatial pattern of expression described here is different from that of ruminants where expression is confined to granulosa tissue of healthy follicles. The pattern is similar to that found in pigs where IGFBP-2 mRNA expression is greater in theca than granulosa tissue.

Previous work in other species has indicated that follicle growth is characterized by a decrease in the concentrations of IGFBP-2 in follicular fluid with a concomitant reduction in expression in granulosa tissue [4]. Our results showed additional intense hybridization associated with a small number of blood vessels, but it remains unclear whether such expression is restricted to a certain size or type of vessel, or between stages of follicular development. An association of IGFBP-2 with blood vessels has been previously reported [5]. The intense hybridization suggests a role for IGFBP-2 in modulating regional blood flow and/or transport of IGFs from the circulation into the thecal tissue.

The functional significance of the higher expression of IGFBP-2 in thecal tissue during the breeding season compared to the vernal transition remains to be determined; however, in addition to regulating thecal cell functions it may also be involved in transporting IGF from the blood vessels through the thecal tissue to the granulosa cells. There was no significant difference in expression of IGFBP-2 mRNA in the granulosa cells of follicles collected during the transition and breeding season indicating that changes in the bioavailability of IGF may not be directly related to reduced estrogen production during the vernal transition.

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Research

Plasma FSH, inhibin A and inhibin isoforms containing pro- and -αC during winter anoestrus, spring transition and the breeding season in mares

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Ten mares were studied from February (winter anoestrus) to their second ovulation in the breeding season to investigate the relationship between resumption of ovarian cyclicity in the spring and circulating concentrations of FSH, inhibin A and inhibin isoforms containing pro- and -αC immunoreactivity. An additional four mares were studied during one oestrous cycle. Growth and regression of ovarian follicles were monitored by transrectal ultrasonography. The frequency of blood sampling varied from three times a week to once a day, depending on the follicular activity present. Concentrations of FSH, oestradiol, inhibin A and pro- and -αC isoforms were low during deep winter anoestrus when minimal follicular activity was present in the ovaries. During spring transition, an increase in FSH concentration preceded the emergence of each follicular wave. Concentrations of inhibins were significantly higher ($P < 0.05$) during growth of anovulatory follicles in spring transition than during winter anoestrus. Plasma concentrations of oestradiol and inhibin A were significantly higher ($P < 0.001$, $P < 0.05$, respectively) during the growth of preovulatory follicles than during the growth of transitional anovulatory follicles, but concentrations of inhibin pro-αC isoforms did not differ between the two types of follicle. During the oestrous cycle, there was a significant inverse relationship ($P < 0.001$) between concentrations of FSH and the inhibins. Plasma inhibin pro-αC isoforms, but not inhibin A, reached a peak on the day of ovulation. The results strongly indicate that FSH regulates growth of spring anovulatory and preovulatory follicles. Inhibins are likely to contribute to negative feedback on the release of FSH from the pituitary gland both during the transitional period and the breeding season in mares.

Introduction

The mare is seasonally polyoestrus with the natural breeding season extending from May to October (Ginther, 1974). During winter anoestrus, the hypothalamo–pituitary axis is essentially non-functional. GnRH secretion is greatly reduced, possibly via dopaminergic inhibition (Besognet *et al.*, 1996), and the pituitary fails to release significant amounts of gonadotrophins (Hart *et al.*, 1984). Pituitary content of LH is low, but the FSH content remains unchanged and there appear to be differences in the proportion and type of pituitary gonadotrophs during anoestrus (Eagle and Tortonese, 2000). In response to the low concentrations of circulating gonadotrophins during winter anoestrus, mares have small, hard ovaries with only one or two follicles with a diameter of < 15 mm.

The period of spring transition is characterized by a resurgence of follicular activity, irregular oestrous behaviour, and resumption of secretion of gonadotrophins and ovarian steroids. Follicles initially grow and regress, but do not exceed 35 mm in diameter (Ginther, 1990). In many mares, waves of anovulatory follicular development proceed, which are characterized by rhythmic growth and regression of large (> 38 mm) dominant follicles. However, there is individual variation among mares, and some mares do not show clear anovulatory follicular waves (Ginther, 1990). The mean total duration from first detection of follicular growth to first ovulation is approximately 60 days (Sharp and Davis, 1993). It has been suggested that the acquisition of steroidogenic competence by follicles is the key to initiation of cyclicity in transitional mares (Sharp and Davis, 1993), and fluid collected from follicles in spring transition contains very low concentrations of oestradiol.

Inhibin is thought to be important in the control of FSH secretion and follicular growth in mares. Immunization against inhibin suppresses plasma concentrations of FSH, and increases both the number of growing follicles and ovulation rates (McCue *et al.*, 1992; McKinnon *et al.*, 1992; Nambo *et al.*, 1998). Inhibins are dimeric proteins comprising one α-subunit and one of two β-subunits (β_A or β_B) forming

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two isoforms, inhibin A (α-β_A) and inhibin B (α-β_B). In addition, high concentrations of a precursor form of the α-subunit, measured as inhibin isoforms containing pro- and -αC immunoreactivity, have been identified in equine plasma (Nagaoka *et al.*, 1999).

Most studies in normal cyclic mares have reported circulating concentrations of immunoreactive inhibin (Bergfelt *et al.*, 1991; Roser *et al.*, 1994; Nagamine *et al.*, 1998). These assays do not distinguish between the biologically active dimeric forms of inhibin and free monomeric α-subunits, which are thought not to be biologically active. The α-subunits may be present in very high concentrations in the circulation, effectively masking the bioactive dimeric forms (McNeilly *et al.*, 1994) and hence also our understanding of the role of the bioactive dimeric inhibin forms. The concentrations of immunoreactive inhibin and inhibin isoforms with pro- and -αC immunoreactivity increase in late dioestrous mares and reach a peak on the day of ovulation (Bergfelt *et al.*, 1991; Roser *et al.*, 1994; Nagamine *et al.*, 1998; Nagaoka *et al.*, 1999). Immunohistochemistry studies in the equine ovary indicate that dimeric inhibin A is secreted mainly by the granulosa and theca cells of large follicles, whereas the granulosa cells of small follicles are thought to secrete inhibin α-subunit (Nagamine *et al.*, 1998).

Inhibins play an important role in folliculogenesis in the mare, but whether changes in concentrations of inhibin are involved in the initiation of ovarian cyclicity in the spring is unknown. The present study is the first to report the presence of inhibin A during the equine oestrous cycle, and the relationship between follicular growth and plasma FSH, inhibin A, inhibin isoforms containing pro- and -αC immunoreactivity, and oestradiol throughout the spring transition and into the breeding season.

Materials and Methods

Ten mares of mixed breeding, weighing 300–450 kg and aged between 3 and 20 years (mean ± SEM; 9 ± 1.6 years), were studied from the beginning of February to the second ovulation of the breeding season in May or June. The study period was subdivided into: (i) the period of deep winter anoestrus, when the ovaries are small and hard and follicles fail to grow to > 15 mm in diameter; (ii) the early transitional period when follicular growth is erratic, with no obvious pattern, and follicles fail to grow to > 30 mm in diameter; (iii) the transitional period when successive anovulatory follicular waves grow and regress with the dominant follicle reaching a diameter of > 30 mm; and (iv) cyclicity. Ovarian activity was monitored three times each week by transrectal ultrasonography until detection of the first follicle with a diameter of 25 mm on the ovaries, when examinations were performed each day until the first ovulation. Ovarian maps were drawn at the time of ultrasonography to aid in follicle identification. Blood samples were collected by jugular venepuncture into evacuated heparinized tubes three times a week until follicles with a diameter of 20 mm were present on the ovaries, at which time blood samples were collected each day until the first ovulation. Examinations and blood sampling resumed on day 14 after ovulation until the second ovulation. The blood samples were centrifuged at 2000 ***g*** for 15 min at 4°C and the plasma decanted and stored at –20°C until used for assay. Blood samples were collected each day in July and August from another four cyclic mares, weighing 320–400 kg and aged between 3 and 10 years, each day during a complete oestrous cycle. The study was performed under the approval of the University of Edinburgh Ethics Committee and project licence obtained under the Home Office Animals (Scientific Procedures) Act 1986.

Hormone assays

Plasma FSH was measured by radioimmunoassay. A highly purified equine FSH (AFP-5022B) was used for standards and iodination. The antiserum (AFP-2062096) was specific for equine FSH and used at a final dilution of 1:100 000. Otherwise, the method was the same as that described by Watson *et al.* (2000). The main crossreactivity of this antiserum was with equine LH (eLH; 2.5%). Crossreactivity with all other hormones tested (eTSH, ePRL, eGH, eCG, hCG) was < 1%. Sensitivity of the assay was 0.5 ng ml^{-1}. Intra- and interassay coefficients of variation were 6.3 and 10.2%, respectively. Displacement curves produced by serial dilutions of plasma and the addition of known amounts of FSH to samples containing low concentrations of FSH were parallel to the standard curve.

Concentrations of inhibin A and inhibin isoforms containing pro- and -αC immunoreactivity were measured using two-site ELISAs as described previously (Groome *et al.*, 1994, 1995; Menon *et al.*, 2000; Riley *et al.*, 2000; Bleach *et al.*, 2001), with some minor modifications to the inhibin A assay. In the inhibin A assay, 8% (w/v) SDS was used, and both the antibody (PPG 14/6) and the streptavidin–alkaline phosphatase conjugate were diluted in casein buffer (Eurogenetics Ltd, Hampton). Standards used for inhibin A were purified dilutions of 32 kDa bovine inhibin A and for inhibin pro-αC immunoreactivity, a highly immuno-purified preparation from human follicular fluid was used. The slopes of the dilution curves with equine plasma were parallel to the standard curve. The intra- and interassay coefficients of variation were 6–7% for inhibin A, and 7.1 and 8.2%, respectively, for inhibin pro-αC. The assay sensitivities were 15 pg ml^{-1} for inhibin A and 6 pg ml^{-1} for inhibin pro-αC.

Plasma oestradiol concentrations were measured as described by Watson *et al.* (2000). Only plasma collected from transitional mares was assayed for oestradiol. Assay sensitivity was 2 pg ml^{-1}. Intra- and interassay coefficients of variation were 4.6 and 7.8%, respectively.

Statistical analyses

Mean concentrations of FSH were calculated in plasma samples collected from mares in deep winter anoestrus

throughout February ($n = 4$ mares) and from mares in early transition, before the onset of anovulatory follicular waves ($n = 9$ mares). Differences were compared using the Mann–Whitney U test. After the onset of anovulatory follicular waves, the highest concentration of FSH measured at the time of wave emergence (at 15–20 mm) was used to compare the differences in one of the anovulatory and ovulatory follicular waves in each mare ($n = 10$) by paired t test. Peak FSH concentrations at the time of follicular wave emergence in spring transition were compared with concentrations measured 2 days later by the paired t test. Data were log transformed before analysis. Follicle sizes were compared using the Student's t test.

Mean concentrations of oestradiol during an anovulatory and ovulatory follicular wave were calculated from concentrations obtained each day when a follicle was between 25 and 40 mm in diameter. During spring transition, concentrations were compared during the growth phase (> 25 mm), and during the 3 days before and after follicular growth. Differences were analysed using the paired t test on log-transformed data.

Mean concentrations of circulating inhibin were calculated for each mare in samples collected once or twice each week in winter anoestrus, before the onset of follicular waves (7–12 samples from nine mares). Because of the small number of samples, it was not possible to compare deep winter anoestrus with early transition. Mean concentrations were also calculated from samples collected each day throughout one anovulatory follicular wave in spring transition, when the mares had an ovarian follicle of 25–40 mm in diameter ($n = 8$ mares) and throughout one ovulatory wave during cyclicity ($n = 12$ mares). Differences in hormone concentrations between groups were examined first by one-way ANOVA to determine whether there were overall significant differences ($P < 0.05$) between the groups. If a significant difference was noted, the Mann–Whitney U test was used. Mean concentrations of inhibin isoforms in oestrus and dioestrus were compared in the four cyclic mares using the paired t test. The relationships between inhibin A, inhibin pro-αC and FSH were investigated by Pearson's correlation. Growth and regression of follicles were analysed by regression analysis.

Results

All mares were in seasonal anoestrus at the start of the study. Only four of the ten mares were classed as being in deep winter anoestrus, and these mares remained in this category throughout February. At the start of the trial, one mare had an anovulatory follicle of > 30 mm in diameter on one ovary, but this mare was not cyclic. Therefore, data were available for nine mares in the period of erratic follicular growth (early transition) between deep winter anoestrus and spring transition when large anovulatory follicles were present. One mare failed to have at least one anovulatory follicular wave before ovulation; therefore, data were included for spring transition from a total of nine mares.

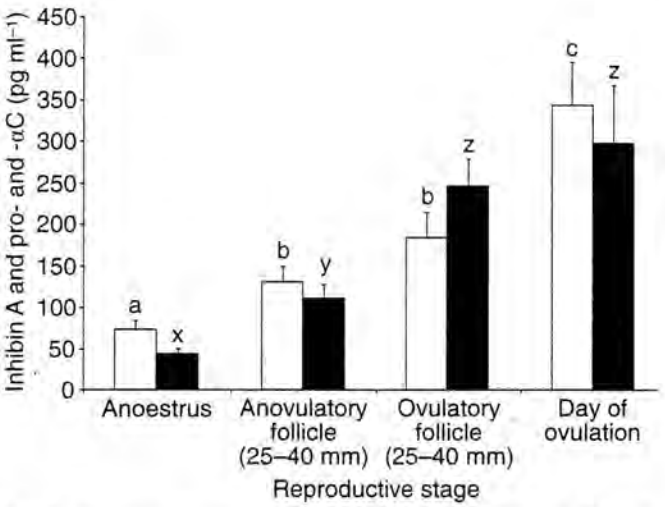

Fig. 1. Plasma concentrations (mean ± SEM) of inhibin A (■) and inhibin pro- and -αC containing isoforms (□) at different reproductive stages in mares. Each column includes data from 8 to 14 mares. [abcxyz]For each hormone, different superscripts represent a significant difference ($P < 0.05$).

Results for cyclic mares include the additional four cyclic mares.

Mean concentrations of FSH were very low during deep winter anoestrus (2.0 ± 0.3 ng ml^{-1}). In early transition, before the onset of anovulatory follicular waves, concentrations of FSH increased significantly (5.1 ± 0.7 ng ml^{-1}; $P < 0.05$) compared with those in deep winter anoestrus. Circulating concentrations of oestradiol, inhibin pro- and -αC isoforms, and inhibin A (Fig. 1) remained low in anoestrus in the presence of small ovarian follicles. At this time there were no significant correlations between inhibins and FSH.

During the spring transition there was an average of 3.7 ± 0.7 follicular waves with dominant follicles of > 30 mm in diameter, at intervals of 9.9 ± 0.8 days. In all nine transitional mares, each follicular wave was preceded by a significant increase ($P < 0.001$) in FSH (7.5 ± 0.8 ng ml^{-1} compared with 4.8 ± 0.5 ng ml^{-1} 2 days later). This increase in FSH was observed when the follicles reached 15–22 mm in diameter (mean = 18.5 ± 0.9 mm; Fig 2). Two days later, the follicles were 20–24 mm in diameter (mean = 22.4 ± 0.4 mm). However, concentrations of FSH did fluctuate during the spring transition and occasionally reached peaks that were not apparently associated with initiation of follicular growth (Fig. 2).

Concentrations of plasma oestradiol were consistently higher ($P < 0.01$) during the growth of an anovulatory follicle (25 mm to peak diameter; 5.1 ± 0.8 pg ml^{-1}) than during the 3 days before and after this period (2.9 ± 0.3 pg ml^{-1}). However, any peak in oestradiol concentrations was short-lived and decreased as soon as the follicle started to regress. During the growth phase of an anovulatory follicle, concentrations of inhibin pro- and -αC containing isoforms were significantly higher ($P < 0.05$) than in anoestrus

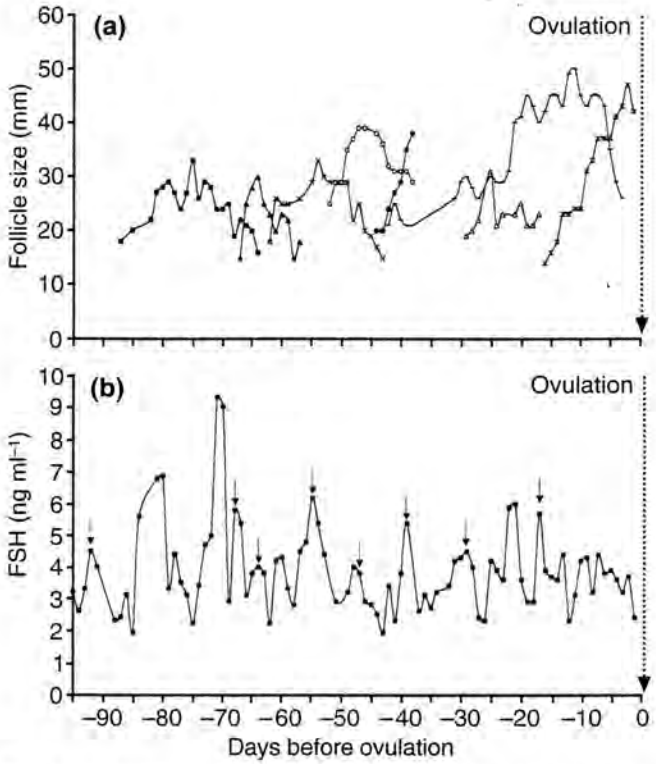

Fig. 2. (a) Follicular waves and (b) plasma FSH concentrations before the first ovulation in one representative mare. Arrows indicate the peaks of FSH corresponding with the emergence of each follicular wave.

(Fig. 1). In spring transition there were negative correlations between plasma inhibin pro-αC isoforms and FSH ($P = 0.06$) and between plasma inhibin A and FSH ($P = 0.1$), which were almost significant.

The size of the largest follicle increased steadily before the first ovulation, and had an increased rate of growth in the final 10 days (Fig. 3a). The number of other large follicles (> 15 mm) decreased rapidly in the 10 days before the first ovulation (data not shown). From day 8 before the first ovulation, the growth of the largest follicle increased linearly ($y = 33.3 + 1.25x$; $R^2 = 92.6\%$; $P < 0.001$), and the second largest follicle regressed ($y = 31.4 - 1.3x$; $R^2 = 73.2\%$; $P < 0.01$). There was an apparent decrease in the mean diameter of the largest follicle between day 10 and day 8 before ovulation because four of the mares had a large regressing anovulatory follicle at the time. The rate of growth of the preovulatory follicle was lower at the first ovulation than at subsequent ovulations ($y = 23.5 + 2.6x$; $R^2 = 96.3\%$; $P < 0.001$; $n = 9$ mares; Fig. 4b).

Mean peak concentrations of FSH at the time of emergence (15–23 mm in diameter; mean = 19.4 ± 1.3 mm) of the first preovulatory follicles (6.6 ± 0.5 ng ml^{-1}) were not significantly different from those measured at the time of emergence of anovulatory follicles for the same mares in spring transition. The peak concentration of FSH was significantly higher ($P < 0.001$) than that measured 2 days later (3.9 ± 0.4 ng ml^{-1}). At this time, the diameter of the dominant follicle was 21–32 mm (mean = 24.9 ± 1.5 mm), which was not significantly different from the growth rate of the anovulatory follicles. Over the 10 days before the first ovulation, circulating concentrations of oestradiol, inhibin A and inhibin pro-αC isoforms increased gradually: oestradiol reached a peak 2 days before ovulation (Fig. 3b), and inhibin pro- and -αC isoforms reached a peak on the day of ovulation (Fig. 3c).

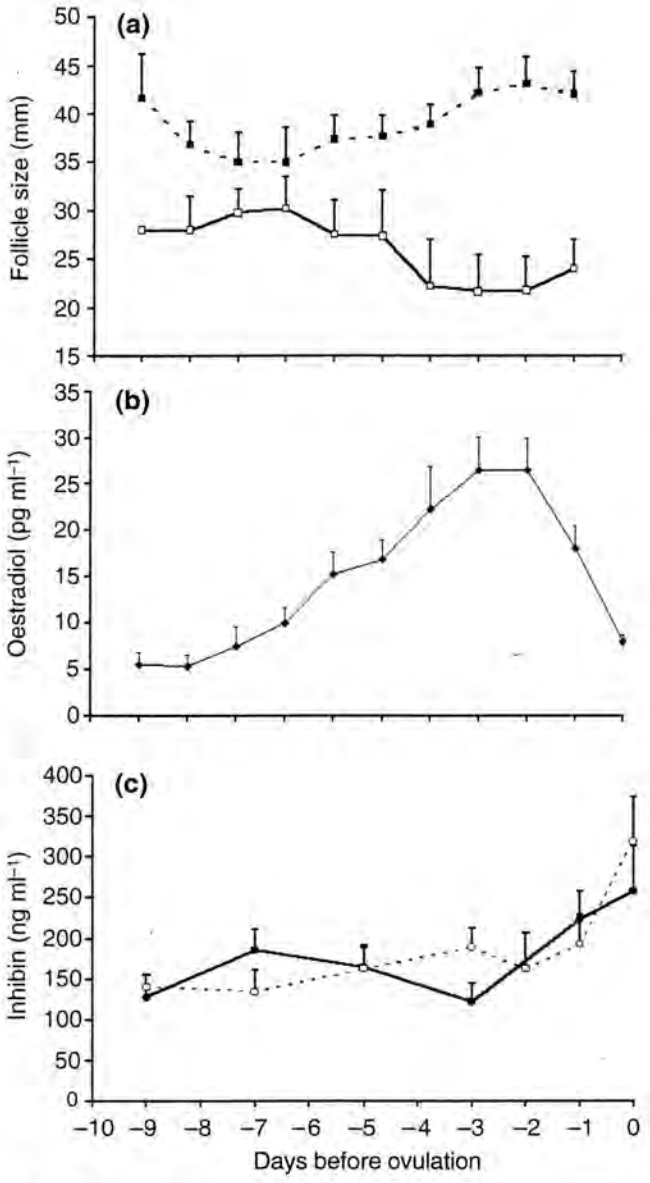

Fig. 3. (a) Growth of the dominant (■) and largest subordinate (□) follicles in mares. Mean (± SEM) concentrations of (b) plasma oestradiol and (c) inhibin A (●) and inhibin pro- and -αC containing isoforms (○) in the 10 days before the first ovulation in mares. Day 0 = day of ovulation.

In cyclic mares, the mean concentration of oestradiol during growth of an ovulatory follicle (19.2 ± 1.8 pg ml^{-1})

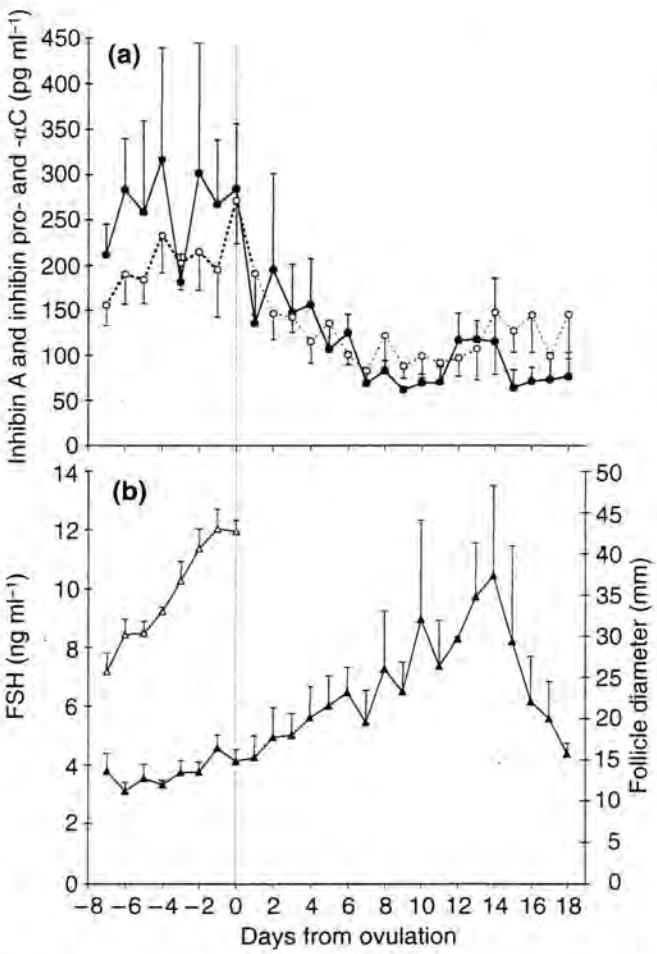

Fig. 4. The normal oestrous cycle of the mare. Each point represents the mean values (± SEM) of 4–14 mares. (a) Plasma concentrations of inhibin A (●) and inhibin pro- and -αC containing isoforms (○), and (b) plasma concentrations of FSH (▲) and follicular growth (△).

was significantly higher ($P < 0.001$) than that during the growth of an anovulatory follicle. Mean concentrations of inhibin pro- and -αC isoforms were highest on the day of ovulation (Fig. 1). Plasma concentrations of inhibin A were significantly higher ($P < 0.05$) during the growth of a preovulatory follicle than during growth of an anovulatory follicle in spring transition (Fig. 1). In contrast to inhibin pro- and -αC isoforms, inhibin A did not reach a peak on the day of ovulation. Concentrations of both immunoreactive inhibin pro-αC and inhibin A were significantly lower ($P = 0.01$) in samples collected each day during dioestrus (101 ± 18.8 pg ml^{-1}; 105 ± 10.5 pg ml^{-1}) than during oestrus (224 ± 48.6 pg ml^{-1}; 392 ± 57.9 pg ml^{-1}) in the four cyclic mares (Fig. 4a). During the oestrous cycle, there was a significant correlation between concentrations of inhibin A and inhibin pro-αC isoforms ($r = 0.870$; $P < 0.001$), and a negative correlation between FSH and inhibin A ($r = -0.655$; $P < 0.001$) and inhibin pro-αC isoforms ($r = -0.657$; $P < 0.001$; Fig. 4a,b).

Discussion

In the present study, there was an average of 3.7 follicular waves with leading follicles of > 30 mm in diameter, at intervals of approximately 10 days during spring transition. This pattern was very similar to that reported by Ginther (1990) and Davis *et al.* (1987). The time of selection and divergence of the future preovulatory follicle, and the decline in the size and the numbers of subordinate follicles in the 8 days before the first ovulation in the present study is agreement with reports by Ginther (1990) and Turner *et al.* (1979). The faster growth rate of the preovulatory follicle at subsequent ovulations compared with the first ovulation has been reported (Ginther, 1990) and is probably due to the higher circulating LH concentrations at subsequent ovulations (Freedman *et al.*, 1979). Circulating LH is thought to be important in the final growth and maturation phase of the follicle in mares (Gastal *et al.*, 2000; Watson *et al.*, 2000).

Concentrations of FSH were low in mares in deep winter anoestrus when the ovaries were small and hard with minimal follicular activity, whereas there was an increase in FSH by the time the mares had significant follicular activity on their ovaries. These findings are in agreement with reports by Turner *et al.* (1979) and Alexander and Irvine (1991). Silvia *et al.* (1987) showed that although concentrations of FSH were lower during deep winter anoestrus, the response to GnRH was greater during deep winter anoestrus than during spring transition or in cyclic mares. These authors concluded that during winter anoestrus, secretion of FSH was low because of reduced secretion of GnRH. In the present study, the emergence of all dominant follicles, anovulatory or ovulatory, was preceded by an FSH surge when the follicle was 15–23 mm in diameter. This finding is similar to that reported during the oestrous cycle by Palmer (1987) and Bergfelt and Ginther (1993). The requirement for FSH in follicle recruitment during the ovulatory season has been reported by Bergfelt and Ginther (1985) and Pineda *et al.* (1973), but the present study is the first to report increases in FSH before each anovulatory wave. The FSH surge in dioestrus reflects higher amplitude FSH pulses (Irvine *et al.*, 1998), and ovulation tended to follow this period of high pulse amplitude within 10 days. More frequent sampling would have been necessary for a detailed profile of FSH concentrations, but from the results of the present study, it seems likely that recruitment is similar in both the spring transition and during the breeding season.

The present study is the first report of the presence of circulating concentrations of inhibin A in mares and inhibin concentrations in the period from winter anoestrus to cyclicity. Previous studies have reported circulating concentrations of immunoreactive inhibin in cyclic mares (Bergfelt *et al.*, 1991; Roser *et al.*, 1994; Nagamine *et al.*, 1998; Nagaoka *et al.*, 1999). The assays used in the present study detect forms of monomeric inhibin (α inhibin) using the inhibin pro- and -αC assay, as well as dimeric inhibin A. Only dimeric inhibin A, not the free α-subunit, is biologically active in other species (Robertson *et al.*, 1986; Knight

et al., 1989). The inhibin β_A subunit is confined to granulosa and theca cells in large equine follicles (Nagamine *et al.*, 1998), and fluid collected from small follicles contained very low concentrations of both inhibin A and inhibin pro-αC immunoreactivity (Tanaka *et al.*, 2000). The absence of large follicles in deep anoestrus may explain the low circulating concentrations of the isoforms of inhibin measured in the present study. Furthermore, in contrast to preovulatory follicles, which produced high concentrations of circulating inhibins, large anovulatory transitional follicles in the mares used in the present study appeared to be producing significantly lower concentrations of dimeric inhibin.

Circulating concentrations of inhibin A and inhibin pro-αC isoforms were higher in oestrus than in dioestrus, but only inhibin pro-αC isoforms increased on the day of ovulation. Studies using a non-selective assay showed that plasma concentrations of immunoreactive inhibin were higher in oestrus than in dioestrus and were at a maximum on the day of ovulation (Bergfelt *et al.*, 1991; Roser *et al.*, 1994; Nagaoka *et al.*, 1999). The peak in circulating concentrations of pro-αC inhibin isoforms may originate from peritoneal absorption of follicular fluid at ovulation (Bergfelt *et al.*, 1991). The present study showed that inhibin A did not increase on the day of ovulation compared with other days during oestrus. Concentrations of inhibin pro-αC isoforms in follicular fluid of large follicles were approximately 100 times higher than those of inhibin A (Tanaka *et al.*, 2000), and this may have been reflected in the circulating concentrations after ovulation. Therefore, it appears that the measured circulating increase in immunoreactive inhibin at ovulation derives predominantly from the α-subunit rather than from dimeric inhibin.

FSH concentrations decrease approximately 8 days before ovulation both in the normal oestrous cycle (Miller *et al.*, 1980; Bergfelt and Ginther, 1993) and before the first ovulation of the breeding season (Freedman *et al.*, 1979; Alexander and Irvine, 1991). In the present study, it was shown that this is the time when circulating concentrations of biologically active dimeric inhibin are increasing, before the first and subsequent ovulations. Furthermore, in previous years in our cyclic pony mares, the largest subordinate follicle did not start to decrease in size until approximately 3 days before ovulation (Pedersen, 2000). This is at a time when circulating concentrations of inhibin A are very high and, therefore, the preovulatory increase in inhibin is temporally associated with regression of the subordinate follicle. Release of FSH does not seem to be closely regulated by GnRH in the breeding season or in winter anoestrous mares (Garza *et al.*, 1986; Silvia *et al.*, 1987). It has been suggested that both during anovulatory follicular waves in spring transition and during growth of the preovulatory follicle, the dominant follicle produces suppressive substances that inhibit the growth of the smaller follicles by negative feedback on FSH at the pituitary. It is thought that these substances may be oestradiol or inhibin (Silvia *et al.*, 1987; Ginther, 1990; Bergfelt and Ginther, 1993). During winter anoestrus, plasma concentrations of FSH, inhibin A and inhibin pro- and -αC isoforms were low. Furthermore, in the presence of anovulatory follicular waves in the spring, concentrations of inhibin A were higher than at anoestrus, but were significantly lower than during the same period of growth of ovulatory follicles. During winter anoestrus, greater variations were seen in FSH than during cyclicity, and so perhaps the absence of suppression by high circulating concentrations of inhibin A permitted intermittently high concentrations of FSH at this time. Furthermore, it is possible that lack of strong negative inhibin feedback at this time may have permitted the higher gonadotrophin response to treatment with GnRH in anoestrous mares compared with that in cyclic mares reported by Silvia *et al.* (1987). It is notable that as transition progressed, the FSH response to GnRH decreased. This corresponds to the increasing concentrations of inhibin measured between anoestrus and cyclicity in the present study.

The present study, in agreement with Davis and Sharp (1991), has shown that anovulatory follicles are not associated with high circulating concentrations of oestradiol. However, there was a significant increase in concentrations of both oestradiol and dimeric inhibin during the anovulatory follicular waves compared with periods when no large follicles were present. Furthermore, there was a significant decrease in FSH as the dominant follicle grew after emergence. Therefore, the combined effect of inhibin and oestradiol, which is more strongly inhibitory than either hormone on its own (Miller *et al.*, 1981), may be important in suppressing the pituitary release of FSH that was measured during spring transition when the dominant anovulatory follicle reached a mean diameter of 22 mm. Donadeu *et al.* (2001) proposed that equine follicles acquire the ability to secrete inhibin with FSH-suppressing capacity by the time they reach 13 mm in diameter. However, the contributory effect of other follicular products cannot be discounted.

The preovulatory increase in inhibin in the present study corresponded well to the emergence of the preovulatory follicle at the time when other follicles regressed. Furthermore, there was a strong negative correlation between inhibin and FSH concentrations during the oestrous cycle in the present study as previously reported using a non-selective assay (Bergfelt *et al.*, 1991; Nagaoka *et al.*, 1999). Oestradiol also has a negative feedback effect on FSH in the mare and reduces the pituitary response to GnRH (Sharp *et al.*, 1991). As in the transitional mare, it is likely that both inhibin and oestradiol contribute to the decrease in FSH. Furthermore, oestradiol has been suggested as a candidate for involvement in the mechanism that leads to deviation in the diameters of follicles in mares (Gastal *et al.*, 1999). It is also possible that inhibin may play a role in deviation, as immunoreactive inhibin increases as early as 9 days before ovulation (Nagamine *et al.*, 1998) or between day 7 and day 12 of the oestrous cycle (Bergfelt *et al.*, 1991). However, inhibins are not thought to play a role in follicle dominance in cattle (Mihm *et al.*, 2000). The role of inhibins in follicle selection in mares requires further study.

In conclusion, it appears that both inhibins and FSH have

an important role in controlling follicle growth during the spring transition as well as during the oestrous cycle in mares. The inverse relationship between FSH and inhibins strongly indicated a negative feedback effect of inhibins on pituitary release of FSH, both during the spring transition and cyclicity. The peak in plasma concentrations of immunoreactive inhibin on the day of ovulation appears to derive from follicular release of inhibin α-subunit isoforms rather than from dimeric inhibin A.

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Changes in the concentrations of steroids and prostaglandin F in preovulatory follicles of the mare after administration of hCG

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Summary. Fluid was aspirated from the preovulatory follicle of Group 1 mares (N = 6) when follicles reached 32–34 mm in diameter. Group 2 mares each received an i.v. injection of hCG when the preovulatory follicle reached 35 mm. Aspiration of follicular fluid was performed 28–32 h after treatment. Follicular fluid was aspirated from Group 3 mares 28–32 h after the preovulatory follicle reached 35 mm in diameter.

Concentrations of progesterone were significantly higher in follicular fluid from Group 2 mares than in that from mares in Groups 1 and 3. Testosterone was significantly higher in follicular fluid from Groups 2 and 3 than in Group 1 mares. There were no significant differences among groups in concentrations of oestradiol and prostaglandin F (PGF) in follicular fluid.

Keywords: oestradiol; progesterone; testosterone; PGF; follicle; mare

Introduction

In many species there is extensive information on concentrations of steroids and prostaglandins (PGs) in follicular fluid in the preovulatory period. In the mare, however, investigations have been limited to collection of follicular fluid from mares slaughtered on certain days of the oestrous cycle or classification of follicular status retrospectively by histological examination.

At the beginning of oestrus, oestradiol is the dominant steroid secreted by the preovulatory follicle of the mare (Kenney *et al.*, 1979; Meinecke *et al.*, 1987). The oestradiol is probably largely synthesized from testosterone which is the main steroidal product of the theca interna from preovulatory follicles (Tucker *et al.*, 1986). Fay & Douglas (1987) reported that concentrations of testosterone in fluid collected from the presumptive preovulatory follicle after ovariectomy significantly increased between Days 1 and 4 of oestrus, whereas concentrations of oestradiol and progesterone did not change; however, because events were timed from the first day of behavioural oestrus, it was not possible to relate hormone concentrations to ovulation.

In other species, concentrations of PGs increase shortly before ovulation and this increase is thought to be mandatory for ovulation (Armstrong *et al.*, 1974; Killick & Elstein, 1987; Munalulu *et al.*, 1987). In mares, elevated concentrations of PGF have been measured in fluid from follicles showing histological evidence of atresia (Kenney *et al.*, 1979) but no serial samples have been collected from preovulatory follicles. Indirect evidence exists, however, for the involvement of PGs in ovulation in the mare. Fenprostalene, a synthetic analogue of PGF with an extended half-life of 24 h, hastened follicular maturation and time of ovulation in mares injected during oestrus (Savage & Liptrap, 1987).

In mares the preovulatory follicle may be identified by ultrasound examination *per rectum* when it is approximately 32 mm in diameter (Pierson & Ginther, 1985). Ovulation occurs at a mean

diameter of 45 mm, 6 or more days later (Pierson & Ginther, 1985). Administration of hCG to mares when the preovulatory follicle is 35 mm in diameter results in ovulation 36–48 h later (Duchamp *et al.*, 1987). Therefore, by using timed follicular puncture after hCG administration, the maturity of the follicle may be assured. In the present study, fluid was aspirated from the preovulatory follicle of oestrous mares by a colpotomy procedure during early follicular growth (32–34 mm), or 28–32 h after the follicle was 35 mm in diameter in mares receiving or not receiving an i.v. injection of hCG. Concentrations of progesterone, oestradiol-17β, testosterone and PGF were measured in the follicular fluid.

Materials and Methods

Animals and follicular aspiration

Animals. Light horse mares, 2–15 years of age, were teased daily with a stallion to determine onset of oestrus. When mares were in oestrus, ovaries were examined daily *per rectum* by palpation and ultrasound to monitor follicular development and measurements of follicular diameter were made using ultrasound. On the first day of oestrus the 18 mares were assigned randomly to one of 3 groups. Fluid was aspirated from the preovulatory follicles of Group 1 mares (N = 6) on the day that follicles first reached 32–34 mm in diameter. Group 2 mares (N = 6) each received an intravenous injection of 2000 i.u. hCG (Butler Co., Columbus, OH, U.S.A.) on the day the preovulatory follicle was first seen to be 35 mm or greater in diameter. Aspiration of follicular fluid was performed between 28 and 32 h after injection. The preovulatory follicles from Group 3 mares (N = 6) were aspirated between 28 and 32 h after the examination at which follicular diameter was first discovered to be 35 mm or greater in diameter.

Follicular aspiration. A colpotomy procedure was used to aspirate follicular fluid (Hinrichs & Kenney, 1987). Mares were sedated by administration of 100 mg xylazine (Bayvet Division Miles Laboratories Inc., Shawnee, KS, U.S.A.) and 20 mg acepromazine (TechAmerica Group Inc., Elwood, KS, U.S.A.) i.v., with 10–15 mg butorphanol (Bristol Laboratories Division of Bristol-Meyers Co., Syracuse, NY, U.S.A.) i.v. as an analgesic. The perineum was scrubbed with a povidone–iodine solution and the operator's hand was introduced into the peritoneal cavity via an incision in the cranial vagina. A trochar was inserted into the peritoneal cavity through the abdominal musculature of the flank. The ovary was manipulated by the hand in the abdomen and the follicular fluid aspirated via a needle inserted through the trochar.

Radioimmunoassays

Oestradiol-17β. The method used has been described by Watson *et al.* (1987). The antiserum (5322) cross-reacted with oestradiol-17β 100%, oestriol 0·6%, oestrone 0·4% and other steroids tested <0·1%. Recovery of radiolabelled oestradiol following a double extraction of 1:10 000 dilution of follicular fluid in ethyl ether was 92%. The within- and between-assay coefficients of variation were 11 and 17% respectively. The assay blank was lower than the lowest standard and sensitivity with reference to the standard curve was 2·5 pg/ml. Accuracy determinations yielded a linear regression of $y = 1{\cdot}33 + 0{\cdot}93x$ and a correlation coefficient of 0·99.

Progesterone. Progesterone was measured using a kit (Diagnostic Products Corporation, Los Angeles, CA, U.S.A.) with ^{125}I-labelled progesterone as a tracer. The assay was modified to include standards (2·5–1000 pg) prepared in assay buffer rather than in the human plasma provided. Samples were diluted 1:20, 1:50 and 1:100 and extracted twice with petroleum ether. The antiserum had the following cross-reactivities: progesterone 100%, 11-deoxycorticosterone 1·7%, 11-deoxycortisol 2·4%, 20α-dihydroprogesterone 2%, 5β-pregnan-3,20-dione 1·3% and other steroids tested <1%. The limit of sensitivity of the assay was 25 pg/ml. The within- and between-assay coefficients of variation were 6 and 9% respectively.

Testosterone. Follicular fluid (0·1 ml) was extracted twice in double-distilled water (1 ml) with methyl-*t*-butyl ether (5 ml) and assayed at 1:10 dilution. Standards were prepared in methanol and ranged from 10 to 500 pg/0·1 ml. [1,2,6,7-^{3}H]Testosterone (0·1 ml; 10 000 cpm; Amersham, Arlington Heights, IL, U.S.A.) was added both to samples and standards, followed by antiserum (Wein Laboratories, Inc., Succasunna, NJ, U.S.A.; lyophilized plasma reconstituted to 35 ml; 0·1 ml per tube). The assay was incubated at 4°C for 2 h. After addition of 0·5 ml charcoal–dextran (0·25% charcoal; 0·025% dextran), samples were incubated for 10 min at 4°C. The tubes were then centrifuged at 2000 *g* for 10 min at 4°C and the supernatant poured into scintillation vials. Scintillation fluid was added (3·5 ml; 4 l toluene:15 g PPO) and the vials counted in a scintillation counter after overnight equilibration. The antiserum had the following cross-reactivities: 5α-dihydrotestosterone 60%, Δ^{1}-testosterone 49%, 5α-androstan -3α,17β-diol 3%, Δ^{5}-androsten-3β,17β-diol 16%, 5α-androstane-3,17-dione 2%, aldosterone 5%, androstenedione 2% and other steroids tested <1%. The assay blank was non-detectable and sensitivity of the assay with reference to the standard curve was 50 pg/ml. Within- and between-assay coefficients of variation were 7 and 10%, respectively. Accuracy determinations yielded a linear regression of $6{\cdot}08 + 0{\cdot}70x$ and a correlation coefficient of 0·99.

PGF. Prostaglandin F was measured directly in follicular fluid and at a 1:5 dilution. The assay method has been described by Watson *et al.* (1987). Standard curves prepared in assay buffer and in follicular fluid showed good parallelism. Their coefficient of correlation was 0·92. Sensitivity of the curve was 0·25 ng/ml. Cross-reactivities of the antiserum (>0·1%) were with 100% PGF-2α, 75% with PGF-1β, and 1·5% with PGF-2β. The within-assay coefficient of variation was 4%. All samples were included in one assay.

Statistical analysis

Concentrations of hormones in different treatment groups were compared using a one-way analysis of variance. To stabilise the variance, data were $\log_{10}$ transformed. Values of $P < 0{\cdot}05$ were considered to be significantly different.

Results

Table 1 shows concentrations of steroids measured in follicular fluid from the three groups of mares. Concentrations of progesterone were significantly higher in follicular fluid from Group 2 than from Group 1 ($P < 0{\cdot}001$) and Group 3 ($P < 0{\cdot}02$) mares but no significant difference existed between Group 1 and Group 3 mares. Testosterone concentrations were significantly higher in follicular fluid from Group 2 than from Group 1 ($P < 0{\cdot}01$) mares. Fluid from Group 3 mares contained significantly higher testosterone concentrations ($P < 0{\cdot}05$) than did that from Group 1 mares. Concentrations of oestradiol in follicular fluid were not significantly different between groups. Concentrations of PGF from all of the Group 1 mares and from all but one of the Group 3 mares were below the detection limit of the assay. No significant differences existed between groups in concentrations of PGF.

Table 1. Concentrations (ng/ml) of progesterone, testosterone, oestradiol and PGF in follicular fluid aspirated from mares in Groups 1, 2 and 3

	Group 1 (N=6)	Group 2 (N=6)	Group 3 (N=6)
Progesterone	20·2 ± 8·82[a]	401·5 ± 146·6[b]	98·3 ± 83·62[a]
Testosterone	5·9 ± 1·02[a]	12·1 ± 1·49[b]	15·0 ± 4·45[b]
Oestradiol-17β	894 ± 166·8	1059 ± 248·4	1160 ± 232·7
PGF	<0·3	18·8 ± 10·84	0·7 ± 0·37

Values are mean ± s.e.m.
Means with different superscripts are significantly different ($P < 0{\cdot}05$) when the data are $\log_{10}$ transformed.

Discussion

The present study shows that, at 28–32 h after treatment with hCG, intrafollicular concentrations of progesterone, testosterone and probably PGF increase. Previous studies have shown that between 82 and 89% of mares with a follicle of 35 mm in diameter and treated with hCG ovulate within 48 h, and most of these will ovulate between 24 and 48 h (Loy & Hughes, 1966; Sullivan *et al.*, 1973; Duchamp *et al.*, 1987). By contrast, only 38% of mares which had a follicle of 34 mm at the start of the study (similar to Group 3 mares), ovulated by 48 h (Duchamp *et al.*, 1987). In the present study, therefore, ovulation was imminent in the hCG-treated mares.

In the mare, previous data showed a tendency for larger preovulatory follicles to contain higher concentrations of progesterone than smaller, less mature follicles (Fay & Douglas, 1987). There is

also histological evidence of luteinization as the follicle matures towards ovulation (Kenney *et al.*, 1979). The present study demonstrated approximately a 20-fold increase in progesterone concentrations as ovulation approached, which is similar in magnitude to the increase reported for preovulatory rat follicles (Munalulu *et al.*, 1987). In mammals, species differences apparently exist for requirement of progesterone for ovulation. In the ewe, progesterone is an intermediary in the action of LH in the mechanism of ovulation (Murdoch *et al.*, 1986) and treatment with antiserum against progesterone decreases ovulation rates in rats (Mori *et al.*, 1977). Progesterone does not appear to be necessary, however, for the ovulatory process in rabbit ovaries perfused *in vitro* (Holmes *et al.*, 1985).

In the mare, as in other species, there is evidence that testosterone is produced by the theca interna under the control of LH (Tucker *et al.*, 1986). The increase in testosterone measured in Group 2 and 3 mares in the present study can therefore probably be attributed to the high circulating concentrations of LH during oestrus, and the large increase in thecal LH receptors in the preovulatory follicle during late oestrus (Fay & Douglas, 1987). Similar results have been reported for other species (Dieleman *et al.*, 1983; Munalulu *et al.*, 1987).

The large increase in the oestrogen-synthesizing ability of the horse follicle occurs when the follicle reaches about 30 mm in diameter (Kenney *et al.*, 1979), which is earlier than the period of study in the present investigation. This study showed that, in the mare, oestradiol concentration in follicular fluid did not increase in the period immediately before ovulation. In women (Lumsden *et al.*, 1986), sheep (Hay & Moor, 1975) and cattle (Dieleman *et al.*, 1983) there is a drop in concentrations of oestradiol in the preovulatory follicle, which may be due to the inhibitory effects of LH on oestradiol synthesis observed *in vitro* (Moor, 1974). The absence of a measurable decrease in oestradiol in horse follicles before ovulation may be due to the difference in the preovulatory LH profile. In the mare, the preovulatory LH surge is relatively prolonged and peak concentrations are not attained until after the time of ovulation (Alexander & Irvine, 1982) whereas, in other species, ovulation occurs several hours after peak plasma LH concentrations.

The results of this study showed that, in the mare, concentrations of PGF tended to increase in follicular fluid before ovulation. It is probable that this pattern reflects gonadotrophin stimulation of PG production from follicular cells (Patwardham & Lanthier, 1981). In other species, rising concentrations of PGs in follicular fluid appear to be necessary for successful ovulation (Armstrong *et al.*, 1974; Murdoch *et al.*, 1986; Killick & Elstein, 1987) by mediating changes in vasculature and collagen degradation and synthesis in the follicle wall (Murdoch & Myers, 1983; Okamuru *et al.*, 1985). The requirement for PGs in the ovulatory process in the mare has not been established.

It will now be necessary to investigate periovulatory hormonal changes in horse follicles in more detail, in association with administration of pharmacological substances directly into the preovulatory follicle to determine factors controlling ovulation.

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Concentrations of arachidonate metabolites, steroids and histamine in preovulatory horse follicles after administration of human chorionic gonadotrophin and the effect of intrafollicular injection of indomethacin

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ABSTRACT

This study investigated the sequence of hormonal changes within the preovulatory follicles of mares. Mares were injected i.v. with 2500 IU human chorionic gonadotrophin (hCG) when a preovulatory follicle of 35 mm in diameter was detected. Fluid was aspirated from preovulatory follicles before (0 h), and 12, 24 and 36 h after administration of hCG. Concentrations of progesterone, prostaglandin (PG) E_2, PGF, 6-keto-$PGF_{1\alpha}$ and thromboxane B_2 in follicular fluid increased significantly ($P<0{\cdot}01$) between 0 and 36 h. At 36 h, PGE_2 was present in highest concentrations, followed by PGF and 6-keto-$PGF_{1\alpha}$; thromboxane B_2 was present at lower concentrations than other prostanoids. Concentrations of 13,14-dihydro-15-keto-$PGF_{2\alpha}$ increased significantly ($P<0{\cdot}05$) between 24 and 36 h. Leukotriene B_4, leukotriene C_4 and histamine were present in follicular fluid at all sampling periods and did not change significantly over time.

In another experiment, buffered saline or indomethacin (either 100 or 500 μg) was injected into preovulatory follicles on the day that they reached 35 mm in diameter to determine whether blocking intrafollicular PG synthesis would affect ovulation. The interval between intrafollicular injection and ultrasonographic detection of luteinization was significantly longer ($P<0{\cdot}05$) in mares treated with 500 μg indomethacin. Plasma progesterone concentrations were significantly ($P<0{\cdot}05$) lower in indomethacin-treated mares than in control mares on the first 5 days after injection.

These results indicate that intrafollicular concentrations of PGs increase significantly before ovulation in mares and may be involved in the ovulatory process.

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INTRODUCTION

In spite of the relative ease with which follicular fluid may be aspirated from mares, there have been few studies on sequential changes in intrafollicular hormone concentrations as the time of ovulation approaches. High concentrations of oestradiol-17β and androgens are present in follicular fluid from preovulatory follicles of mares (Short, 1962; Kenney, Condon, Ganjam & Channing, 1979; Fay & Douglas, 1987). Concentrations of progesterone and testosterone (but not oestradiol) in fluid collected from preovulatory follicles significantly increased shortly before ovulation (Watson & Hinrichs, 1988).

Concentrations of prostaglandins (PGs) in follicular fluid increase as ovulation approaches in pigs (Ainsworth, Tsang, Downey *et al.* 1979), rats (Munalulu, Hillier & Peddie, 1987), rabbits (Koos, Clark, Janson *et al.* 1983), sheep (Murdoch, Dailey & Inskeep, 1981) and man (Lumsden, Kelly, Templeton *et al.* 1986). Studies have shown that if this increase is prevented by administration of indomethacin, the follicle does not ovulate but luteinizes and functions as a corpus luteum (Grinwich, Kennedy & Armstrong, 1972; Ainsworth *et al.* 1979; Murdoch & Dunn, 1983; Killick & Elstein, 1987). These results have led to the suggestion that PGs are involved in mediating ovulation (LeMaire & Marsh, 1975; Espey, 1980; Murdoch & Cavender, 1987). In the mare, there is little information on the importance of PGs in the ovulatory process. In preovulatory follicles, PGF tended to increase 28 to 32 h after administration of

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human chorionic gonadotrophin (hCG) (Watson & Hinrichs, 1988). Administration of a synthetic analogue of $PGF_{2\alpha}$ hastened ovulation in the mare (Savage & Liptrap, 1987), although another study, using a different preparation, showed no effect (Squires, Harrison, McKinnon & Voss, 1988). Systemic administration of dexamethasone, a PG inhibitor, blocked ovulation in the mare, but plasma concentrations of luteinizing hormone (LH) were also reduced (Asa & Ginther, 1982), therefore, it is not possible definitively to implicate PG inhibition in failure of ovulation.

Products of the 5-lipoxygenase route of arachidonate metabolism and histamine have also both been implicated in the process of ovulation. Inhibition of 5-lipoxygenase has resulted in failure of ovulation in rats (Reich, Kohen, Naor & Tsafriri, 1983; Reich, Kohen, Slager & Tsafriri, 1985) and administration of antihistamines has blocked ovulation in rabbits (Wallach, Wright & Hamada, 1978; Knox, Lowry & Beck, 1979) but not in ewes (Halterman & Murdoch, 1986) or gilts (Hall, Meisterling, Lewis & Daily, 1989). There is no information on concentrations of these substances in preovulatory follicles in mares.

It is possible to manipulate the time of ovulation in the mare by i.v. administration of hCG when the preovulatory follicle reaches 35 mm in diameter. Ovulation occurs between 36 and 48 h later (Duchamp, Bour, Combarnous & Palmer, 1987), which is approximately 3 days sooner than it would occur in mares not treated with hCG (Pierson & Ginther, 1985). The present study measured the concentrations of hormones and histamine in follicular fluid aspirated at set intervals after administration of hCG to mares with preovulatory follicles of 35 mm in diameter. The effect of injection of indomethacin, a cyclooxygenase inhibitor, into follicles 35 mm in diameter was studied by monitoring the time to ovulation and luteinization (by ultrasonography) and subsequent luteal function (by plasma progesterone analysis).

MATERIALS AND METHODS

Animals

Fourteen Standardbred and Thoroughbred mares aged 3 to 16 years were used. The mares were teased daily with a stallion to detect onset of oestrus. From the first day of oestrus, the ovaries were examined by ultrasonography and the diameter of the preovulatory follicle was recorded.

Experiment 1

Aspiration of follicular fluid

On the day on which a 35 mm follicle was detected (days 2–5 of oestrus), 12 of the mares were injected in the jugular vein with 2500 IU hCG (Butler Co., Columbus, OH, U.S.A.). The mares were divided randomly into three groups of four and follicular fluid was aspirated either immediately after the 35 mm follicle was detected (group 1), or after 12 h (group 2) or 24 h (group 3). At the next oestrus, four of the mares from groups 1, 2 and 3 were selected at random to form group 4 and fluid was aspirated from their follicles 36 h after detection of a 35 mm follicle and administration of hCG. Because aspiration of follicular fluid induces subsequent luteinization (McKinnon, Carnevale, Squires *et al.* 1988), each mare could only be sampled once during each oestrous cycle. Two follicles reached 35 mm on the same day in one mare from group 2, in two mares from group 3 and in one mare from group 4. In each instance, fluid from both follicles was aspirated and the data were included in the analysis.

Experiment 2

Injection of indomethacin

Thirteen of the mares were used. When a 35 mm follicle was detected (days 2–5 of oestrus), hCG (2500 IU) was injected into the jugular vein and either 2 ml 0·1 mol phosphate-buffered saline/l (PBS; four mares) or 100 μg (four mares) or 500 μg (five mares) indomethacin (Sigma Chemical Co., St Louis, MO, U.S.A.) prepared in 2 ml sterile PBS was injected directly into the follicle. At the time of injection, no change in follicular volume or tension was detected by the operator. Time to ovulation/luteinizing of the follicle (the corpus luteum in the mare is recognizable from the day of ovulation by its characteristic echogenic appearance) was monitored by ultrasonography (using the criteria described by Townson & Ginther, 1988, 1989*a*) performed at 12-h intervals. Blood samples were collected each day by jugular venepuncture into heparinized evacuated tubes until return to oestrus. The blood was centrifuged at 2000 ***g*** for 10 min and the plasma stored at −20 °C until assayed for progesterone.

Aspiration of follicular fluid and injection of indomethacin

The procedure for aspiration of follicular fluid was described by Watson & Hinrichs (1988). Briefly, after appropriate sedation and analgesia, a stab incision was made into the peritoneal cavity via the area of the flank over the ovary. The operator's hand was inserted into the peritoneal cavity via an incision in the cranial vagina. An 18 gauge, 18 cm spinal needle was then inserted through the stab incision in the flank. The stilette was removed from the needle. The ovary was manipulated by the hand in the abdomen and the needle inserted into the preovulatory follicle.

Follicular fluid was aspirated into a 60 cm^3 syringe attached to a sterile polypropylene extension tube. The entire fluid content of the follicle was removed (approximately 30–35 ml). If blood-tinged fluid appeared in the tube, aspiration was stopped before this fluid reached the syringe. Injection into the follicles was performed using the same technique as that employed for aspiration. PBS (2 ml) or indomethacin (2 ml) were drawn into a syringe and injected into the follicle via the extension tube. This volume was chosen to ensure delivery of the entire dose into the follicle and was not anticipated to disrupt the structure of the 35 mm follicle (already containing 35 ml fluid). Before injection, the plunger of the syringe was withdrawn slightly until follicular fluid was visible in the extension tube to ensure that the needle was located within the follicle.

Treatment of follicular fluid

The follicular fluid was stored on ice in polypropylene tubes until transported to the laboratory. The fluid was centrifuged within 10 min of collection at 2000 ***g*** for 10 min at 4 °C to pellet any aspirated granulosa cells and was then dispensed into 2 ml polypropylene vials and stored at −70 °C until assay.

Radioimmunoassays

Oestradiol-17β

Oestradiol-17β was measured in follicular fluid by radioimmunoassay after extraction with ethyl ether, as described previously (Watson & Hinrichs, 1988). Within- and between-assay coefficients of variation were 11 and 17% respectively. Sensitivity of the assay was 6 pmol/l plasma and accuracy determinations yielded a regression equation of $y = 1{\cdot}33 + 0{\cdot}93x$ with a correlation coefficient of 0·99. Ninety-one per cent radiolabelled oestradiol was recovered from follicular fluid.

Progesterone

Progesterone was measured in follicular fluid by radioimmunoassay using Coat-a-Count tubes (Diagnostic Products Co., Los Angeles, CA, U.S.A.) after the fluid was extracted with petroleum ether (Watson & Hinrichs, 1988). Sensitivity of the assay was 0·8 nmol/l plasma. All samples were included in one assay. Within-assay coefficient of variation was 6%. Recovery of progesterone added to follicular fluid was $93 \pm 4{\cdot}8$% over the range 8–31 nmol/l. Progesterone was assayed directly in blood plasma (Watson & Hinrichs, 1989) using the same reagents as for follicular fluid. Standards were prepared in ovariectomized mare plasma. Sensitivity of the assay was 0·8 nmol/l. Within- and between-assay coefficients of variation were 6 and 9% respectively.

Testosterone

Follicular fluid was extracted twice in ethyl ether. The extract was reconstituted in ovariectomized mare plasma and assayed for testosterone using Coat-a-Count tubes from Diagnostic Products Co. Standard concentrations of testosterone were also prepared in ovariectomized mare plasma. Main cross-reactivities of the antibody were with 5α-dihydrotestosterone, 3·3%; 19-nortestosterone, 20%; 11-ketotestosterone, 16%; methyltestosterone, 1·7%; 11-β-hydroxytestosterone, 1%; androstenedione, 0·5%; and 5-β-androstan-3α,17-β-diol, 0·4%. Accuracy determinations yielded a regression equation of $y = 1{\cdot}01x + 1{\cdot}78$ with a correlation coefficient of 0·99. Sensitivity of the assay was 0·3 nmol/l. Within-assay coefficient of variation was 17%. Ninety-seven per cent of radiolabelled testosterone was recovered from follicular fluid. All samples were included in one assay.

PG assays in follicular fluid

The direct radioimmunoassay of PGF in follicular fluid has been described previously (Watson & Hinrichs, 1988). Owing to the high cross-reactivity of the antiserum with $PGF_{1\alpha}$, the results are expressed as total immunoreactive PGF. Sensitivity of the assay was 0·89 nmol/l. Within-assay coefficient of variation was 4%. PGE_2, 6-keto-$PGF_{1\alpha}$ and thromboxane B_2 were measured directly in follicular fluid using reagents purchased from New England Nuclear (Boston, MA, U.S.A.). Antisera cross-reactivities have been described previously (Watson & Sertich, 1990). Sensitivity of the PGE_2 assay was 8·8 pmol/l. Within-assay coefficient of variation for PGE_2 was 6·5% (at 35 pmol/l) and 11·0% (at 178 pmol/l). Sensitivity of the assay was 68 pmol/l. The within-assay coefficient of variation for 6-keto-$PGF_{1\alpha}$ was 6%. Sensitivity of the assay for the thromboxane B_2 was 37 pmol/l. Within-assay coefficient of variation was 15%. For all PGs, standard curves prepared in assay buffer and in follicular fluid showed good parallelism (coefficient of correlation $> 0{\cdot}99$).

Concentrations of 15-keto-13,14-dihydro-$PGF_{2\alpha}$ (PGFM) were measured in follicular fluid using an extraction assay similar to that described previously for plasma (Watson, Stokes, David *et al.* 1987) in which follicular fluid was extracted with ethyl ether. Sensitivity of the assay was 0·18 nmol/l. The within-assay coefficient of variation was 14%. Recovery of radiolabelled PGFM was 110%. For each PG, all samples were included in one assay.

Leukotriene assays in follicular fluid

Concentrations of leukotriene B_4 (LTB_4) and leukotriene C_4 (LTC_4) were measured by radioimmunoassay using reagents purchased from New England Nuclear. Follicular fluid was acidified and extracted

by previously described methods (Isono, Koshihara, Murota *et al.* 1985; Katsura, Minamisawa, Katayama *et al.* 1988) using pretreated Sep-Pak C_{18} cartridges (Waters Associates, Milford, MA, U.S.A.). The extract was resuspended in assay buffer before assay. Cross-reactivities of the leukotriene B_4 antiserum were with 5,12-dihydroxyeicosatetraenoic acid, 3·6%; 20-OH-LTB_4, 1·3%; 6-trans LTB_4, 1·0%; LTD_4, 11·6% and LTE_4, 3·3%; and of the LTC_4 antiserum were with LTB_4, 0·03%. Sensitivities of the assays were 0·4 nmol LTB_4/l and 1·6 nmol LTC_4/l. Within-assay coefficients of variation were 8% for LTB_4 and 10% for LTC_4. Recovery of LTB_4 and LTC_4 after the extraction procedure was 89% and 82% respectively. All samples were included in one assay.

Histamine

Histamine was measured by a radioenzymatic assay using radioenzymatic-grade histamine N-methyl-transferase. Reagents were purchased from New England Nuclear. The assay was performed using methods described previously (Verburg, Bowsher & Henry, 1983). Level of sensitivity of the assay was 90 pmol/l. Intra-assay coefficient of variation was 8% and all samples were included in one assay.

Statistical analysis

Hormone data were $\log_{10}$ transformed before analysis. Differences in hormone concentrations at different time-intervals were determined by a one-way analysis of variance (due to unequal numbers of mares in the groups). Where appropriate, means were compared using least significant difference. Values are given as means ± S.E.M.

RESULTS

Experiment 1

At the time of aspiration, follicle diameters were 36±0·7 mm (group 1), 36±0·5 mm (group 2), 36±1·3 mm (group 3) and 36±2·6 mm (group 4).

Concentrations of steroid hormones in follicular fluid at intervals after hCG injection are shown in Fig. 1. There were no significant differences in concentrations of oestradiol or testosterone after injection of hCG. Progesterone concentrations increased significantly between 0 and 36 h.

Concentrations of prostanoids are shown in Fig. 2. In general, there was little change in prostanoid concentrations until after 24 h. Between 24 and 36 h, concentrations of all prostanoids increased significantly. Concentrations of PGFM in follicular fluid were 1·2±0·4 nmol/l at 0 h, 0·6±0·2 nmol/l at 12 h, 0·3±0·06 nmol/l at 24 h and 1·6±0·4 nmol/l at 36 h. Concentrations increased significantly ($P<0·05$) between 12 and 36 h and between 24 and 36 h. LTB_4, LTC_4 and histamine were detectable in all samples of follicular fluid. There were no significant changes in

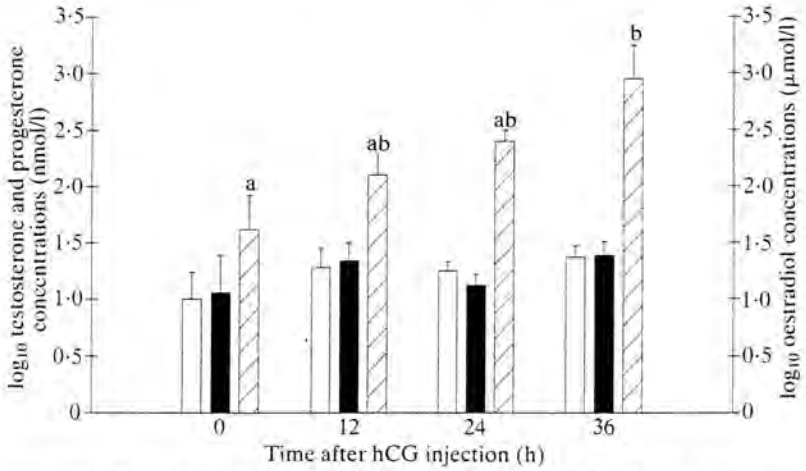

FIGURE 1. Mean ± S.E.M. concentrations of testosterone (open bars), oestradiol-17β (solid bars) and progesterone (hatched bars) in follicular fluid before and 12, 24 and 36 h after injection of human chorionic gonadotrophin (hCG) into mares. Values with a different letter are significantly different ($P<0·01$) by analysis of variance followed by least significant difference comparison of means. Four mares were included in each group and the number of follicles aspirated were four at 0 h, five at 12 h, six at 24 h and five at 36 h after administration of hCG.

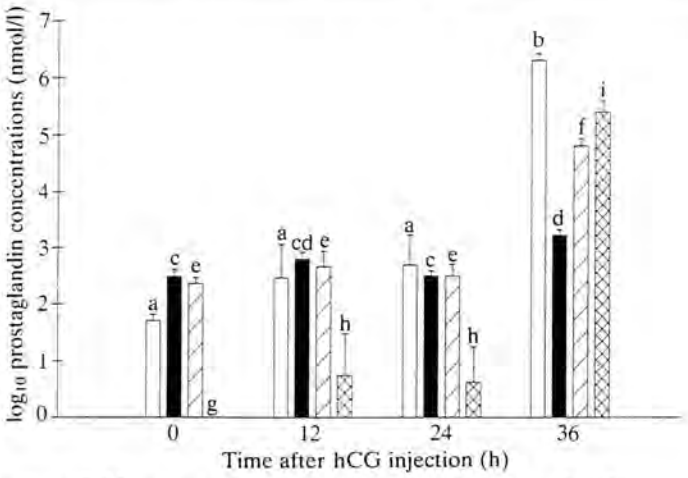

FIGURE 2. Mean ± S.E.M. concentrations of PGE_2 (open bars), thromboxane B_2 (solid bars), 6-keto-$PGF_{1\alpha}$ (hatched bars), and PGF (cross-hatched bars) in follicular fluid at 0, 12, 24 and 36 h after administration of human chorionic gonadotrophin (hCG) to mares with preovulatory follicles 35 mm in diameter. Values with a different letter are significantly different ($P<0{\cdot}01$) by analysis of variance followed by least significant difference of means. Four mares were included in each group and the number of follicles aspirated were four at 0 h, five at 12 h, six at 24 h and five at 36 h after administration of hCG.

TABLE 1. Mean ± S.E.M. concentrations of leukotriene B_4 (LTB_4), leukotriene C_4 (LTC_4) and histamine in follicular fluid collected 0, 12, 24 and 36 h after administration of human chorionic gonadotrophin (hCG) to mares with preovulatory follicles of 35 mm in diameter

Time after administration of hCG (h)	No. of follicles	LTB_4 (μmol/l)	LTC_4 (nmol/l)	Histamine (nmol/l)
0	4	1·0 ± 0·1	8·1 ± 1·5	0·28 ± 0·05
12	5	1·4 ± 0·3	5·6 ± 0·2	0·18 ± 0·05
24	6	1·2 ± 0·1	7·5 ± 1·3	0·17 ± 0·04
36	5	0·9 ± 0·1	6·3 ± 2·8	0·33 ± 0·05

concentrations of LTs or histamine between 0 and 36 h after injection of hCG (Table 1).

Experiment 2

Mares treated with 500 μg indomethacin took longer ($P<0{\cdot}05$) to develop ultrasonographically detectable luteal tissue (48, 48, 60, 72 and 84 h after injection) than mares treated with PBS (12, 12, 48 and 48 h). Results for mares treated with 100 μg indomethacin were not significantly different from either group (12, 24, 36 and 84 h).

Plasma progesterone concentrations (Table 2) were significantly ($P<0{\cdot}05$ or $P<0{\cdot}01$) lower in treated mares than in control mares during the first 5 days after injection (day 0 = day of follicular injection). After day 5, concentrations were not significantly different among groups. Length of cycle (day of injection to return to oestrus) was not significantly different for mares treated with PBS (19 ± 0·6 days), 100 μg indomethacin (18 ± 0·8 days) or 500 μg indomethacin (18 ± 0·9 days).

DISCUSSION

We have shown that between 24 and 36 h after administration of hCG to mares with preovulatory follicles

TABLE 2. Mean ± S.E.M. concentrations of plasma progesterone (nmol/l) in mares after injection of PBS, 100 μg or 500 μg indomethacin into 35 mm preovulatory follicles

	Treatment with PBS ($n=4$)	Treatment with indomethacin	
		100 μg ($n=4$)	500 μg ($n=5$)
Day after injection			
1	1·3 ± 0·75	<0·8**	0·6 ± 0·50*
2	3·1 ± 0·60	<0·8**	0·3 ± 0·44
3	3·8 ± 0·31	1·3 ± 0·75**	2·2 ± 0·60*
4	7·2 ± 0·79	3·8 ± 2·20**	3·8 ± 0·38**
5	10·4 ± 1·32	10·0 ± 4·14	5·7 ± 0·66*

For each day, values with asterisk are significantly lower than the controls. *$P<0·05$, **$P<0·01$ compared with control (analysis of variance with least significant difference mean comparisons).

of 35 mm in diameter, there were large increases in intrafollicular concentrations of PGF, PGE_2 and 6-keto-$PGF_{1\alpha}$. A significant but less dramatic increase in thromboxane B_2 and progesterone was also seen. There were no significant changes in concentrations of other substances measured.

The increase found in progesterone concentrations in the present study is in agreement with a previous study, in which follicular fluid from preovulatory follicles of mares contained significantly higher concentrations of progesterone 28 to 32 h after administration of hCG than at the time of administration of hCG (Watson & Hinrichs, 1988). Other studies in mares have also provided evidence that follicle cells luteinize before ovulation (Younglai, 1972; Kenney *et al.* 1979; Tucker, Henderson & Duby, 1988).

Testosterone is produced by the theca interna cells of horse follicles under the control of LH (Tucker, Henderson, Duby & Morcom, 1986). In a previous study, when follicular fluid samples were collected at the time of administration of hCG or 28 to 32 h later, a significant increase in testosterone concentrations was observed as the follicle approached the time of ovulation (Watson & Hinrichs, 1988). By contrast, in the present study, no significant changes in concentrations of testosterone were detected over the sampling period; concentrations were relatively high throughout the entire period. It is possible that there was a transient rise in concentrations between 24 and 36 h which was not revealed by our sampling regimen. The high concentrations of testosterone in preovulatory follicles measured in this and other studies (Fay & Douglas, 1987; Meinecke, Gips & Meinecke-Tillmann, 1987) can be attributed to the high circulating concentrations of LH during oestrus and the large numbers of LH receptors in the preovulatory follicle during oestrus (Fay & Douglas, 1987). These high concentrations of testosterone may reflect a decrease in aromatase activity, as has been reported in bovine (Dieleman, Kruip, Fontijne *et al.* 1983) and human (Hillier, Reichert & Van Hall, 1981) preovulatory follicles.

Oestradiol-17β is produced in mares by the granulosa cells of the follicles (Channing, 1969; Seamans & Sharp, 1982). The high concentrations of oestradiol-17β measured in preovulatory follicles in the present study were similar to those described previously (Kenney *et al.* 1979; Meinecke *et al.* 1987; Fay & Douglas, 1987; Watson & Hinrichs, 1988). Concentrations of follicular oestradiol-17β did not decrease immediately before ovulation, in contrast to women (Lumsden *et al.* 1986), sheep (Hay & Moor, 1975) and cattle (Dieleman *et al.* 1983). Concentrations of oestradiol in mare follicular fluid is reported to decline between mid- and late oestrus (Tucker *et al.* 1988), so perhaps, in our study, samples were obtained after the fall in concentrations had occurred.

Results of studies in many species suggest that ovulation is an inflammatory process involving increased intrafollicular concentrations of PGs, hyperaemia and oedema (Ainsworth *et al.* 1979; Espey, 1980; Murdoch & Myers, 1983; Okuda, Okamura, Kanzaki *et al.* 1983). In the present study, increases in intrafollicular prostanoid concentrations were not detected until 24 to 36 h after hCG had been administered. At 36 h after administration of hCG, PGE_2 was present in the highest concentrations, followed by PGF and 6-keto-$PGF_{1\alpha}$. There was no evidence that, as in the ewe, PGF was synthesized preferentially to PGE_2 as the time of ovulation approached (Murdoch, Peterson, Van Kirk *et al.* 1986) nor was there evidence of a nadir in PG concentrations 24 h after administration of hCG, as was found in women (Lumsden *et al.* 1986). In our study, 6-keto-$PGF_{1\alpha}$ increased significantly as ovulation approached, but Murdoch *et al.* (1986) were unable to detect increases in follicular 6-keto-$PGF_{1\alpha}$ around ovulation in the ewe. Thromboxane B_2 was present in lower concentrations than the other prostanoids measured in horse follicles. Concentrations of the $PGF_{2\alpha}$ metabolite PGFM decreased within the first 24 h. It is possible, therefore, that the increased concentration of follicular PGF resulted from decreased metabolism rather than increased synthesis. However, between 24 and 36 h, concentrations of PGFM increased, probably due to the very high intrafollicular concentrations of PGF. At 0 h, follicular fluid contained high concentrations of PGFM and yet concentrations of PGF were very low. This study did not reveal what intrafollicular events may have occurred before the sampling period to account for this paradoxical finding. Interestingly, increases in PG concentrations during the preovulatory period in the ewe were confined to the follicular tissue, and concentrations in follicular fluid and ovarian

venous plasma remained unchanged (Murdoch *et al.* 1981). In pigs (Ainsworth *et al.* 1979; Tsang, Ainsworth, Downey & Armstrong, 1979) and rabbits (Triebwasser, Clark, LeMaire & Marsh, 1978), as in the mare, high concentrations of PGs are found in follicular fluid.

Leukotrienes B_4 and C_4 were present in preovulatory mare follicles but did not increase over time after administration of hCG. Similarly, in ewes it was concluded that LTs were not a key element in ovulation (Carvalho, Yeik & Murdoch, 1989).

The release of histamine is thought to be a significant event during ovulation. In our study, there were no significant changes in histamine concentrations in the follicular fluid of mares between 0 and 36 h after administration of hCG. It is possible that samples were collected after the period during which changes in concentrations of intrafollicular histamine were taking place.

Blockade of ovulation by administration of indomethacin is well documented in other species (Ainsworth *et al.* 1979; Murdoch & Dunn, 1983; Schmidt, Holmes, Owman *et al.* 1986; Killick & Elstein, 1987) and is dependent upon inhibition of intrafollicular PG concentrations (Murdoch, 1989). After inhibition of ovulation, oestrous cycle length is not affected. The follicles enlarge, luteinize and persist for the lifespan of normal corpora lutea. In the present study, luteinization of the preovulatory follicle visualized by ultrasonography tended to be delayed in mares that had received 500 μg indomethacin. Owing to technical difficulties and the ovarian anatomy of the mare, ovulation was not confirmed by laparoscopy or oviducal flushes. In women treated with indomethacin to induce luteinized unruptured follicles, plasma progesterone concentrations during early dioestrus were lower than in control women, but concentrations in both treated and control women were similar later in dioestrus (Killick & Elstein, 1987). Interestingly, these results in women parallel the findings in the present study. The decreased progesterone concentrations early in the cycle confirm the ultrasonographic diagnosis of delayed ovulation/luteinization in mares treated with indomethacin. It is interesting to speculate that indomethacin treatment may have interfered with the hyperaemia and angiogenesis that have been observed in the ovulating follicle and in the developing corpus luteum of the ewe (Cavender & Murdoch, 1988). Any early reduction in blood supply may have reduced circulating concentrations of progesterone.

Although the average diameter of the mare follicle at ovulation is 45 mm (Pierson & Ginther, 1985), in our study, follicles were 36 mm in diameter 36 h after administration of hCG. This finding agrees with a previous study in which follicles did not grow significantly between administration of hCG and ovulation (Townson & Ginther, 1989*b*). In experiment 1, two follicles appeared to be ready to ovulate during four of the 16 oestrous periods. In each instance, both follicles were judged to be preovulatory. There are few data on hormonal criteria for classification of equine follicles as preovulatory or atretic but Kenney *et al.* (1979), using histological features to classify follicles, suggested that high concentrations of oestrogens were anti-atretogenic. In the four mares in our study with two apparently preovulatory follicles, concentrations of oestradiol-17β in follicular fluid were high in both of the follicles. In cattle, potentially ovulatory follicles had higher concentrations of oestradiol than of the other steroids, whereas atretic follicles contained higher concentrations of progesterone and androgens than of oestradiol (Ireland & Roche, 1982). On the basis of these criteria, in our mares, all follicles sampled fitted into the potentially ovulatory category.

In conclusion, the present study showed that concentrations of prostanoids and progesterone increased in preovulatory horse follicles prior to ovulation. Treatment of mares with indomethacin delayed ultrasonographically detectable luteinization and decreased plasma progesterone concentrations in early dioestrus. Future work should show whether administration of indomethacin to preovulatory horse follicles blocks the preovulatory rise in prostanoid concentrations.

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Detection of chemotactic factors in preovulatory follicular fluid from mares

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SUMMARY

Ovulation has been likened to an inflammatory process. Inflammatory cells accumulate in the ovulating follicle, presumably because of chemotactic factors. Chemotactic activity was measured in fluid aspirated from follicles of estrous mares 0, 12, 24, and 36 hours after ultrasonographic detection of a 35-mm follicle and IV treatment with 2,500 IU of human chorionic gonadotropin. Chemotaxis was assessed by measuring directional migration of equine neutrophils under agarose. Follicular fluid acted as a chemoattractant for neutrophils, but there was no significant difference in chemotactic activity among different time intervals after administration of human chorionic gonadotropin. On the basis of results of various treatments, chemotactic properties of serum and follicular fluid were similar. Chemotactic activity was significantly reduced by heating (56 C for 30 minutes) and by trypsinization and was virtually removed by charcoal treatment. Dialyzing the follicular fluid (3,500 and 8,000 molecular weight cut-off) significantly reduced the chemotactic activity of follicular fluid and serum. The importance of chemotactic factors in the process of ovulation in the mare is yet to be established.

The process of ovulation has been described as an acute inflammatory response[1] in that concentrations of prostaglandins and other inflammatory mediators in follicular fluid increase as time of ovulation approaches in sows,[2] rats,[3] rabbits,[4] ewes,[5] women,[6] and mares. Around the time of ovulation, inflammatory cells accumulate in the follicle, changes occur in the vasculature of the ovary, proteolytic enzymes are activated, and concentrations of intrafollicular histamine, prostaglandins, and steroids increase. It has recently been suggested that inflammatory cell infiltration may have a role in ovulation in the ewe through release of substances that mediate tissue breakdown[7,8] The presence of these leukocytes indicates that chemotactic substances are present within follicles. Chemotactic activity has been detected in vitro in follicular fluid from women[9,10,a] and in medium conditioned by ovine follicles.[8] A link has been reported between chemotactic factors in follicular fluid and optimal conception rates in stimulated in vitro fertilization cycles in women[9] and it was suggested that the presence of these factors might indicate maturity of the follicles. In agreement with these data, the chemotactic properties of ovine follicles increased with time after the preovulatory surge of luteinizing hormone in plasma.[8]

The purpose of the study reported here was to test the chemotactic activity of fluid aspirated from mare preovulatory follicles at serial times after administration of human chorionic gonadotropin (HCG) and to investigate the nature of the chemotactic substances(s).

Materials and Methods

Mares—Twelve Standardbred and Thoroughbred mares were used. The experiment was performed in May and June, that is, during the physiologic breeding season. From the first day of estrus, the mares' ovaries were examined daily by ultrasonography. It is possible to manipulate timing of ovulation in the mare by IV administration of HCG when the preovulatory follicle reaches 35 mm in diameter.[11] Ovulation occurs between 36 and 48 hours later. On the day the preovulatory follicle reached 35 mm in diameter, the mares were injected IV with 2,500 IU of HCG. Each mare was randomly assigned to 1 of 3 groups (n = 4). The follicular fluid was aspirated immediately after detection of a 35-mm follicle, or after 12 or 24 hours. At the next estrus, 4 mares were selected at random and the process was repeated, but this time, follicular fluid was aspirated 36 hours after detection of a 35-mm follicle and administration of HCG.

Two follicles reached 35 mm in diameter on the same day in 1 mare in the group in which aspiration was performed at 12 hours, in 2 mares in the 24-hour group, and in 1 mare in the 36-hour group. In each case, follicular fluid was aspirated from both follicles.

Aspiration of follicular fluid—Follicular fluid was aspirated as previously described.[12] After IV administration of 100 mg of xylazine,[b] 20 mg of acepromazine,[c] and 10 to 15 mg of butorphanol,[d] an incision was made in the

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[a] Cummins JM, Seow WK, Thong YH, et al. Partial characterization of a leucotactic factor from human follicular fluid (abstr). *Clin Reprod Fertil* 1987;5:277.
[b] Bayvet Division, Miles Laboratories Inc, Shawnee, Kan.
[c] TechAmerica Group Inc, Elwood, Kan.
[d] Bristol Laboratories, Division of Bristol-Myers Co, Syracuse, NY.

cranial portion of the vagina and a hand was passed into the peritoneal cavity to manipulate the ovary. A 15-cm spinal needle (18 gauge) was inserted into the follicle via a stab incision in the flank and the fluid was aspirated into a syringe attached to the needle by extension tubing.

Follicular fluid samples were kept on ice until they were transported to the laboratory. The samples were centrifuged at 2,000 × g for 10 minutes at 4 C and the supernatants were stored in aliquots at −70 C.

Chemotaxis assay—Chemotactic activity of follicular fluid (4 pooled 24-hour samples) was assessed by measuring directional migration of equine leukocytes under agarose as previously described.[13] The pooled serum control was collected from 2 healthy stallions. Leukocytes were isolated from the blood of a healthy stallion by hypotonic lysis of erythrocytes. The cells were washed 3 times and resuspended in HEPES-buffered Hanks balanced salt solution (HBSS, pH 7.3). Viable cells that excluded trypan blue dye were counted by use of a hemacytometer. Cells were differentiated by use of a stained[e] cytospin preparation; 84 ± 0.7% of cells isolated were neutrophils. Cells were finally diluted to a concentration of 2 × 10^7 neutrophils/ml. The agarose plates were prepared by mixing a hot 1.5% solution of agarose containing 0.4% bovine serum albumin with an equal volume of 2 × HBSS. The solution was poured into 60-mm diameter cell culture dishes and refrigerated at 4 C to increase gel firmness. A template (ID, 5 mm) was used to punch 3 sets of 3 linearly arranged wells (5 mm apart) on each plate. Follicular fluid or equine serum (30 µl) was placed in the middle well and cells (30 µl) were placed in the 2 outer wells. After a 2.5-hour incubation at 39 C in an atmosphere of 5% CO_2:95% air, cells were fixed by the addition of methanol. After 30 minutes, the agarose was removed and the cells were stained.[e] Distance migrated was measured by use of an eyepiece graticule grid (8 squares × 8 squares) on a microscope. The leading front of cells was designated as the last line of squares that contained at least 1 leukocyte in each of 5 separate squares. Any random migration detected on the side of the well opposite to the chemoattractant was subtracted from the distance of directional migration.

To determine what substance(s) might be responsible for the chemotactic activity of follicular fluid, pooled follicular fluid (n = 4 × 24-hour samples) and serum samples were heat treated, dialyzed, extracted with charcoal, and trypsinized.

Heat treatment—Samples were treated at 56 C for 30 minutes.

Charcoal treatment—Samples containing 33 mg of charcoal/ml were incubated at 23 C for 30 minutes with frequent mixing. The charcoal was removed by centrifugation at 2,000 × *g* for 10 minutes at 4 C. Supernatants were filter-sterilized (0.45 µm) before use.[14]

Dialysis—Sample was loaded into dialysis tubing of 3,500 and 8,000 molecular weight (mol wt) cut-off and dialyzed against HBSS for 24 hours at 4 C. The medium was changed twice during this period.

[e] Diff-Quik, American Scientific Products, McGaw Park, Ill.

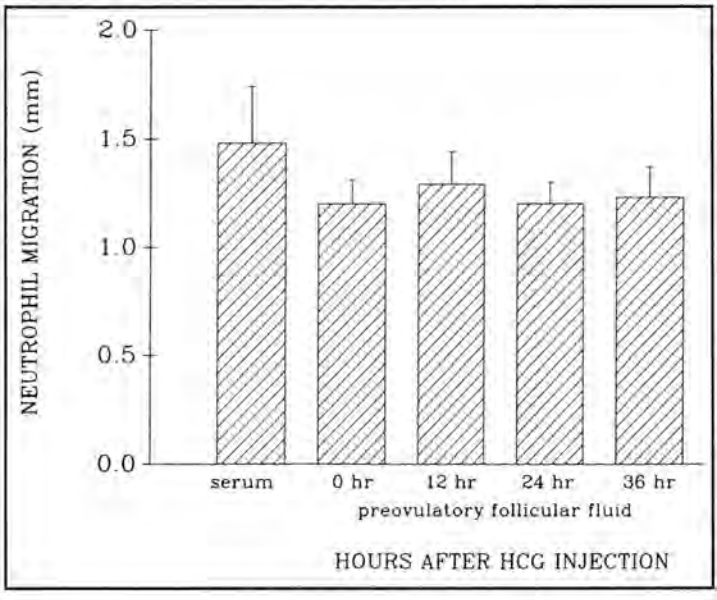

Figure 1—Chemotactic activity of serum and fluid from preovulatory follicles (diameter ≥ 35 mm) of horses (mean of individual samples of follicular fluid ± SEM) collected at 0 (n = 4), 12 (n = 5), 24 (n = 6), or 36 (n = 4) hours after injection of human chorionic gonadotropin (HCG).

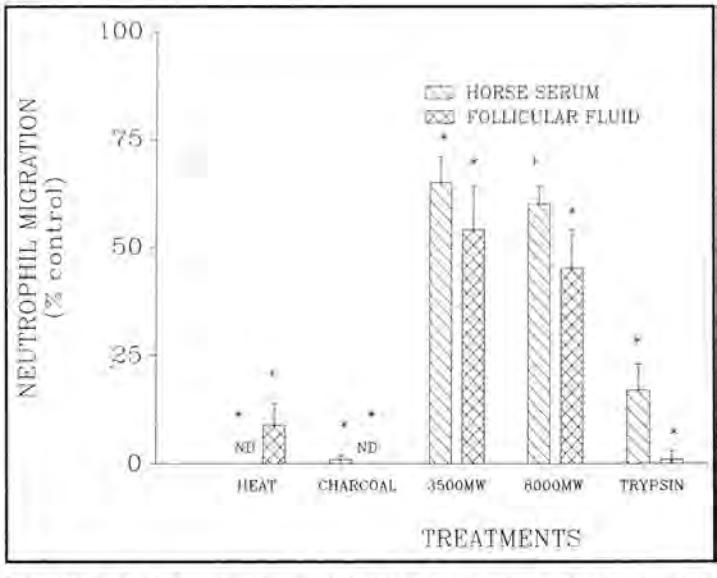

Figure 2—Effect of various treatments on chemotactic activity of serum and follicular fluid (mean ± SEM distance migrated). ND = no detectable migration. * Significantly ($P < 0.01$) different from control. Trypsin treatment significantly reduced chemotactic activity of follicular fluid more than horse serum ($P<0.05$). With the other treatments, there were no significant differences in reduction in chemotactic activity between horse serum and follicular fluid.

Trypsin treatment—Samples containing 200 µg of trypsin/ml[f] were incubated at 37 C for 4.5 hours in a shaking water bath. The enzyme reaction was terminated by adding a twofold excess turkey egg white trypsin inhibitor.[14,f]

Statistical analysis—Individual determinations were replicated 6 times. Differences in distances migrated were analyzed by analysis of variance or repeated measures analysis of variance. Where appropriate, differences be-

[f] Sigma Chemical Co, St Louis, Mo.

ween group means were analyzed by least-significant difference.

Results

Follicular fluid was a chemoattractant for leukocytes nd its chemotactic activity was not significantly differ- nt from that of serum (Fig 1). There was no significant lifference in chemotactic activity of follicular fluid sam- les collected at various time intervals after administra- ion of HCG (samples were tested individually for hemotactic activity). The chemotactic activity of follic- ılar fluid was negligible at dilutions greater than or equal o 1:10 (data not shown). Heat treatment significantly (P < 0.01) reduced chemotactic activity of serum and follic- ılar fluid (Fig 2) and charcoal treatment virtually re- noved the chemotactic activity from serum and follicular luid. Activity was reduced (P < 0.01) in serum and fol- icular fluid after dialysis by use of membranes having a ,500 or 8,000 mol wt cut-off. Trypsinization of serum and ollicular fluid greatly reduced (P < 0.01) chemotactic ctivity. Trypsin treatment reduced chemotactic activity nore in follicular fluid than in serum (P < 0.05). There vere no significant differences between serum and follic- ılar fluid in response to the other treatments.

Discussion

In our study, chemotactic factors were detected in preo- ulatory follicular fluid aspirated from mares with folli- les > 35 mm in diameter, but chemotactic activity did ot increase with time after administration of HCG. In vomen and ewes, appearance of chemotactic activity is ssociated with follicular maturity.[8,9] It is possible that hemotactic activity is already maximal by the time the reovulatory follicle is 35 mm in diameter in the mare. he origin of these chemotactic factors is not clear. Fol- icular fluid is a serum-based fluid with protein compo- ition similar to serum, but the presence of the blood- ollicular barrier and products secreted by granulosa cells neans that the proportions of proteins and other sub- tances may differ.[15] Tissue collected from preovulatory vine follicles has been cultured in vitro and has been hown to secrete chemotaxins.[8] Therefore, it is likely that oth serum and secretions from follicular tissue contrib- ted to the chemotactic activity observed in follicular fluid rom the mares in our study.

In this study, the chemotactic activity of serum and ollicular fluid was affected in a similar manner by var- ous treatments, although trypsinization reduced che- notactic activity of follicular fluid more than serum. imilar chemotactic factors may be implicated in both luids, but proteins may assume a more important role in ollicular fluid or the different proportions of proteins in erum and follicular fluid[15] may cause the differences seen fter trypsinization.

Several substances appeared to be involved in attract- ng neutrophils toward follicular fluid from mares. Tryp- inization of the equine follicular fluid greatly reduced eutrophil migration, thus proteins or protein fragments f various molecular weights may be acting as chemo- ttractants. It has been suggested that the chemotactic omponents of ovine follicular fluid are small peptides hat represent degradation products of collagen produced during ovulation.[8] In addition, a protein or proteins with leukotactic activity have also been identified in follicular fluid from women.[a] Similar factors may be active in equine follicles. In the study by Murdoch et al,[8] dialysis of the follicular fluid by use of a membrane with a 3,000 mol wt cut-off removed chemotactic activity, indicating that the peptides were of low molecular weight. However, the leukotactic protein(s) in follicular fluid of women had a 50,000 mol wt.[a] Mare follicular fluid appeared to contain both low and higher molecular weight chemotaxins.

Heating at 56 C for 30 minutes greatly reduced or removed chemotactic activity of mare serum and follicular fluid. The heat-labile, proteinaceous complement component C5a is known to be an important chemotactic substance in serum[16] and our findings suggest that it may also be important in follicular fluid. Complement component C5a has a 11,000 mol wt. Dialysis of equine follicular fluid by use of a membrane with an 8,000 mol wt cut-off reduced chemotaxis, therefore, lower molecular weight heat-labile chemotactic substances must also be present. Low molecular weight substances are important chemoattractants for neutrophils. Leukotriene B_4 and certain prostaglandins are chemotactic for equine neutrophils.[13] Leukotriene B_4 and prostaglandins have been detected in ovine and human follicular fluid,[17,18] and both are present in high concentrations in equine follicular fluid,[19] therefore, these substances could contribute to the chemotactic activity of follicular fluid. Concentrations of steroids are also high in preovulatory follicular fluid in the mare.[12] Charcoal treatment virtually removed chemotactic activity from serum and follicular fluid and both steroid hormones and arachidonate metabolites would have been removed by charcoal treatment. However, it has been reported that there was no correlation between follicular fluid concentrations of ovarian steroids and the migratory response of human leukocytes.[9] Although systemic treatment of ovariectomized mares with ovarian steroids directly influences migration of equine neutrophils in vitro,[20] to our knowledge, there are no reports of a direct chemotactic effect of ovarian steroids for equine neutrophils.

In conclusion, the chemotactic properties of serum and follicular fluid were similar. It seems likely that proteins and arachidonate metabolites are chemotactic factors in follicular fluid. Further studies will determine the importance of leukotactic factors in the process of ovulation in the mare.

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Immunosuppressive properties of follicular fluid from preovulatory horse follicles

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Summary. Fluid was aspirated from the preovulatory follicles of mares before and 12, 24 and 36 h after intravenous administration of hCG. Follicular fluid significantly ($P < 0{\cdot}001$) reduced lymphocyte blastogenesis *in vitro* and, at a dilution of 1:100, fluid collected at 36 h after administration of hCG was significantly more suppressive ($P < 0{\cdot}01$) than fluid collected before 36 h. Suppression of blastogenesis was reduced by extracting the follicular fluid with ether or by charcoal treatment ($P < 0{\cdot}01$) or by heating at 56°C for 30 min ($P < 0{\cdot}05$). Preincubation of lymphocytes with 2 of 5 follicular fluid samples expressed subsequent blastogenesis. Follicular fluid inhibited blastogenesis of T-cell growth factor (TCGF)-dependent Con A lymphoblasts ($P < 0{\cdot}05$) and the degree of inhibition was related to time of addition of the TCGF and time of collection of the follicular fluid. These results indicate that preovulatory follicular fluid in the mare is increasingly suppressive to lymphocytes as time of ovulation approaches and that this immunosuppression is associated with an alteration of the response to lymphokine stimulation.

Keywords: mare; immunosuppression; follicular fluid

Introduction

Both the oocyte and fertilized ovum are potentially antigenic to the maternal immune system and may be susceptible to the mare's immune defence mechanisms. As the process of ovulation is believed to be an inflammatory response (Espey, 1980), it is important that the inflammatory reaction be curbed before it proceeds to an immune response. In several species, including the horse, antibodies to the zona pellucida have been detected and can prevent spermatozoa from attaching and penetrating ova (Ownby & Shivers, 1972; Glass & Hanson, 1974; Trounson *et al.*, 1980; Shivers & Liu, 1982). It has also been shown that antibodies directed against the zona pellucida can inhibit shedding of the zona by the embryo (Dudkiewicz *et al.*, 1975).

The preovulatory follicle in the mare ovulates when it reaches a mean diameter of 45 mm (Pierson & Ginther, 1985). At ovulation, the infundibulum surrounds the ovary to facilitate passage of the oocyte to the ampulla. This means that, in addition to the oocyte, approximately 45 ml follicular fluid theoretically will pass into the oviduct. It seems possible, therefore, that substances are normally present in follicular fluid which protect the oocyte and developing embryo by suppressing the maternal immune system. Ultrasonographic results suggest that much of the evacuated fluid does not collect in the ipsilateral oviduct (Townson & Ginther, 1989), but the oviduct in ovulating mares was not positively identified. Follicular fluid from women (Fahmi *et al.*, 1985) and cattle (Castilla *et al.*, 1988) suppresses mitogen-induced lymphocyte blastogenesis *in vitro* and in women this mechanism appears to operate via induction of suppressor cell activity (Castilla *et al.*, 1988). Suppressor/cytotoxic lymphocytes (T8) have also been detected in follicular fluid from women (Hill *et al.*, 1987).

The present study investigated the effect on mitogen-induced lymphocyte blastogenesis of plasma or of follicular fluid collected from preovulatory follicles of mares at different intervals after administration of hCG. T-cell growth factors (TCGF), which are mainly interleukin-2 (IL-2) (Oldham & Williams, 1984), are produced by activated T lymphocytes to induce clonal proliferation of T cells. By adding a source of TCGF to activated lymphocytes, it was possible to test whether follicular fluid interfered with TCGF-induced blastogenesis.

Materials and Methods

Animals and experimental design. Normally cycling, light breed horse mares aged between 3 and 16 years were used. Oestrus was detected by use of a teaser stallion. From the first day of oestrus, each mare's ovaries were examined by ultrasonography. On the day that the preovulatory follicle reached 35 mm, 2500 i.u. hCG (Butler Co., Columbus, OH, USA) were administered intravenously. Follicular fluid was aspirated either before administration of hCG or 12, 24 or 36 h later. Follicular fluid from 4 mares was aspirated at each time interval: 1 of the mares at 12 h, 1 at 36 h and 2 at 24 h had two preovulatory follicles of equivalent size. In these mares, fluid was aspirated from both follicles. At the time of aspiration, blood was collected from the jugular vein into a heparinized evacuated tube. Plasma was stored at −20°C.

Aspiration of follicular fluid. Follicular fluid was aspirated using a technique described previously in which, after appropriate sedation and analgesia of the mare, the ovary was held by a hand inserted into the abdomen via an incision in the cranial vagina and the fluid aspirated via a needle inserted through the flank (Hinrichs & Kenney, 1987). After collection, the follicular fluid was stored on ice until centrifuged at 2000 *g* for 10 min at 4°C. The supernatant was then stored in aliquants at −70°C. None of the samples contained significant red blood cell contamination.

Follicular fluid treatments. Follicular fluid collected from follicles 36 h after administration of hCG was subjected to various treatments: (1) 25 mg charcoal was added to 0·75 ml follicular fluid and incubated at room temperature for 30 min with frequent agitation. The mixture was then centrifuged at 2000 *g* for 1 h at 4°C and the supernatant removed and added to a lymphocyte blastogenesis assay. (2) The fluid was heated at 56°C for 30 min before being included in the assay. (3) The fluid (1 ml) was extracted twice with ethyl ether (15 ml). The extract was resuspended in 1 ml medium and both the extract and the follicular fluid were added to a lymphocyte blastogenesis assay. Blastogenesis results were compared with wells containing medium which had been subjected to the same treatments.

Lymphocyte blastogenesis assay. Lymphocytes were isolated from blood of a healthy mare and the assay performed as previously described (Watson, 1990). Follicular fluid (100 μl), undiluted and at dilutions of 1:20 and 1:100 or plasma collected at the time of aspiration (100 μl; 1:20) was added to wells containing lymphocytes (1×10^5) and concanavalin A (Con A 50 μl, 20 μg/ml; Sigma Chemical Co, St Louis, MO, USA). Control wells were included in which medium was substituted for follicular fluid or for Con A. After 72 h incubation, 0·5 μCi [^{3}H]thymidine (sp. act. 6·7 Ci/mmol: New England Nuclear, Boston, MA, USA) was added to each well. The wells were harvested 6 h later. Each follicular fluid or plasma sample was added to wells in quadruplicate.

TCGF production by mitogen-treated cells. Peripheral blood mononuclear cells were isolated as peviously described (Watson, 1990) and were cultured with 80 μg Con A/ml for 2 h as described by Oldham & Williams (1984). Control cultures were performed in the absence of Con A. Cells were washed twice before being incubated for an additional 24 h. The medium was then harvested by centrifugation at 2000 *g* for 10 min and the supernatant stored in aliquants at −70°C.

TCGF assay. TCGF-dependent cells were produced as previously described (Oldham & Williams, 1984) by culturing mononuclear cells with Con A (5 μg/ml) for 4 days. Cells were washed twice and resuspended at 2×10^5/ml before being used in the assay. Serial 2-fold dilutions of the TCGF cell supernatants (100 μl) plus 100 μl medium were added to triplicate cultures of cells (50 μl). A 1:2 dilution of the TCGF supernatant was added to wells containing cells plus follicular fluid (100 μl). After a 24-h incubation, [^{3}H]thymidine (1 μCi) was added to each well and harvested after a further 18 h. Control wells were included in which medium replaced the TCGF supernatant. In another assay, TCGF supernatant was added 6 h after the start of the assay.

Induction of suppressor cells. Mononuclear cells were preincubated for 48 h at 37°C in an humidified atmosphere of 5% CO_2:95% air with a 1:2 dilution of follicular fluid aspirated 36 h after hCG. Controls were included in which follicular fluid was replaced by complete medium (RPMI-1640 containing 10% fetal calf serum, 10^{-5} M-2-mercaptoethanol, 20 mM-Hepes, 2 mM-L-glutamine, 50 units penicillin/ml, 50 μg streptomycin/ml). The cells were then washed twice and resuspended at 2×10^6/ml. These cells (50 μl) were then added to wells containing freshly prepared autologous blood mononuclear cells (50 μl, 2×10^6/ml) and included in a lymphocyte blastogenesis assay.

Determination of cell viability. In each assay, wells were included in which [^{3}H]thymidine was replaced by complete medium. At the end of the assay, the viability of the cells was assessed by exclusion of trypan blue dye.

Statistical analyses. Degree of suppression of [^{3}H]thymidine incorporation was expressed as a percentage of c.p.m. in control wells. A one-way or repeated measures analysis of variance was performed to analyse effects of treatment.

When appropriate, means were compared using the least significant difference equation. Results were considered significant when $P < 0.05$.

Results

Follicular fluid undiluted and at a dilution of 1:20 significantly suppressed lymphocyte blastogenesis (Fig. 1). At a dilution of 1:100, only fluid collected 36 h after hCG was significantly immunosuppressive. Undiluted follicular fluid was equally suppressive at all collection times. At a 1:20 dilution, follicular fluid collected at 36 h was more suppressive ($P < 0.05$) than fluid collected at 12 h and tended ($P < 0.1$) to be more suppressive than fluid collected at 0 and 24 h. When diluted to 1:100, follicular fluid collected at 36 h was significantly ($P < 0.01$) more suppressive than fluid collected at other time intervals. Viabilities of cells suspended in undiluted follicular fluid were 76·3 ± 5·4% in 0 h, 92·0 ± 3·7% in 12 h, 90·0 ± 1·5% in 24 h, and 88·0 ± 2·8% in fluid collected 36 h after administration of hCG. Viability in medium was 86·7 ± 2·9%. Addition of plasma to wells only slightly suppressed blastogenesis ($0.05 < P < 0.1$) compared with control wells (0 h, 73·1 ± 2·9%; 12 h, 91·7 ± 6·5%; 24 h, 87·1 ± 6·1%; 36 h, 76·4 ± 7·4%). Although ether extraction, charcoal stripping, and heat treatment reduced suppression of blastogenesis by follicular fluid (21 ± 3·9%, $P < 0.01$; 16·1 ± 2·3%, $P < 0.01$; and 11·9 ± 3·3%, $P < 0.05$; respectively), the fluid still significantly suppressed blastogenesis ($P < 0.001$). When cells were preincubated in follicular fluid for 48 h before assay (Table 1), 2 of the 5 follicular fluid samples collected 36 h after administration of hCG induced suppressor cell activity. Blastogenic response to TCGF was inhibited

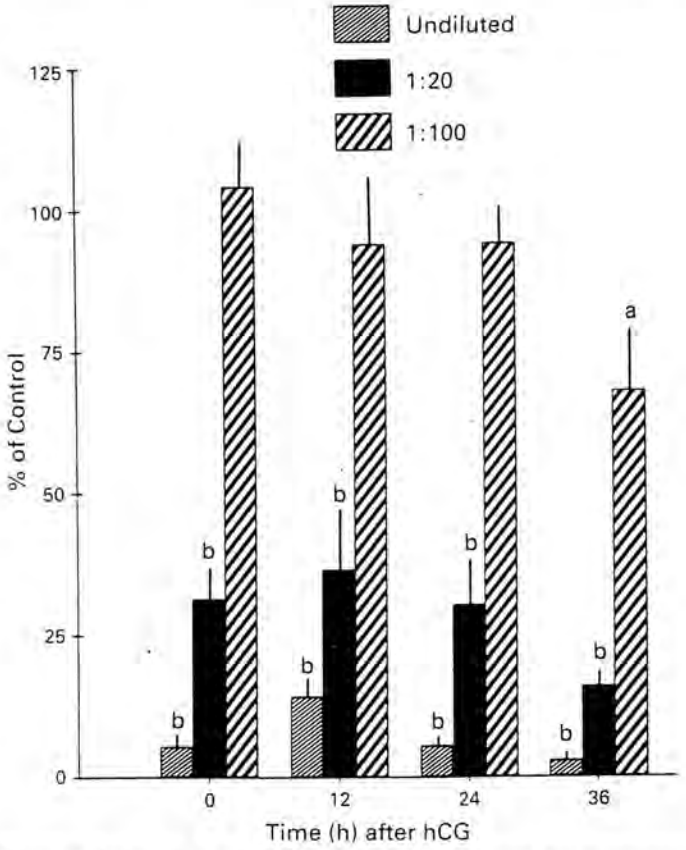

Fig. 1. Effect of follicular fluid on Con A-induced lymphocyte blastogenesis. Follicular fluid collected 0 ($n = 4$), 12 ($n = 5$), 24 ($n = 6$), and 36 ($n = 5$) h after administration of hCG was added to wells undiluted and at dilutions of 1:20 and 1:100. Results are expressed as % of control wells which did not contain follicular fluid. Values with superscripts are significantly different from controls. a, $P < 0.01$; b, $P < 0.001$.

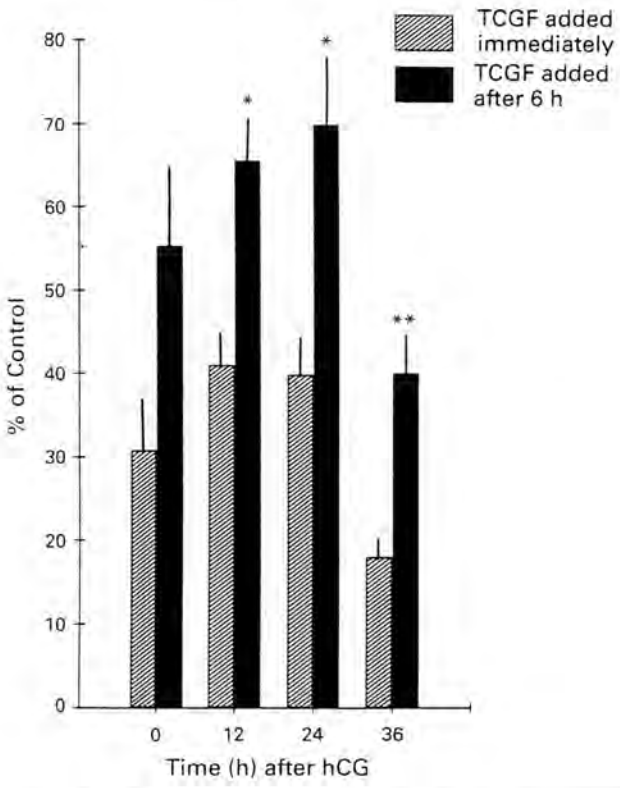

Fig. 2. Effect of follicular fluid on TCGF-induced lymphocyte blastogenesis. Follicular fluid was added to wells and supernatant containing TCGF was then added immediately or 6 h after the beginning of the assay. Results are expressed as % of control wells which did not contain follicular fluid. $*P < 0{\cdot}05$; $**P < 0{\cdot}01$. Significant difference in immunosuppression between addition of TCGF at the start of the assay or 6 h later.

Table 1. Effect of preincubation of cells for 48 h with follicular fluid on subsequent blastogenesis

Preincubation medium	Incorporation of [^{3}H]thymidine (c.p.m.)
Follicular fluid 1	26 227 ± 639
Follicular fluid 2	27 022 ± 598
Follicular fluid 3	14 339 ± 1261[a]
Follicular fluid 4	19 907 ± 1261
Follicular fluid 5	18 734 ± 1322[b]
Medium	22 363 ± 1566

Values are the mean ± s.e.m. of 4 replicate wells.
Figures with superscripts are significantly different from control. a, $P < 0{\cdot}001$; b, $P < 0{\cdot}05$.

($P < 0{\cdot}001$) after addition of follicular fluid (Fig. 2). When addition of TCGF was delayed by 6 h, immunosuppression was reduced in wells containing follicular fluid collected at 12, 24 and 36 h compared with wells in which TCGF was added at the start of assay.

Discussion

This study showed that fluid collected from preovulatory horse follicles suppressed lymphocyte blastogenesis, apparently via alteration of the IL-2 system. When hCG is administered to mares which have a follicle approximately 35 mm in diameter in their ovaries, ovulation normally occurs within 36–48 h (Duchamp *et al.*, 1987). The present results show that the degree of immunosuppression by follicular fluid increased as time of ovulation approached. Previous work with cattle (Fahmi *et al.*, 1985) showed that large follicles possessed more immunosuppressive activity than did small follicles and preovulatory follicles, but there appears to be no information on fluid from maturing preovulatory follicles in any species.

Follicular fluid is formed in part by transudation from serum (Edwards, 1974; Andersen *et al.*, 1976). Little immunosuppressive activity was present in plasma and no differences were detected between samples collected at different time intervals after administration of hCG. It therefore seems likely that, as in the cow (Fahmi *et al.*, 1985), the lymphocyte inhibitory factors are either locally produced or selectively transported from plasma into the follicles.

Measurements of lymphocyte viability revealed similar survival rates between cells cultured in follicular fluid and in control wells containing complete medium. The immunosuppressive effect was therefore not mediated by lymphocyte cytotoxicity. Follicular fluid collected from women suppressed the proliferative response of autologous lymphocytes by inducing or enhancing suppressor cell activity (Castilla *et al.*, 1988). Such activity was apparent in only 2 of 5 follicles when mare follicular fluid was preincubated with lymphocytes before their inclusion in a lymphocyte blastogenesis assay. Differences between individual cows in immunosuppressive factors in preovulatory follicular fluid have been reported by Fahmi *et al.* (1985) and these workers suggested that it was advisable to study samples from individual animals, rather than pooled samples, around the time of ovulation.

It appeared that follicular fluid interfered with TCGF induction of lymphocyte blastogenesis. When addition of TCGF was delayed by 6 h, suppression of lymphocyte blastogenesis was significantly decreased. This suggests that follicular fluid was not merely binding TCGF directly, making them unavailable for lymphocyte stimulation, or masking IL-2 receptors. The follicular fluid could have been acting to suppress protein synthesis within the lymphocytes, reducing IL-2 receptor expression and blastogenesis. If the action of follicular fluid was to decrease TCGF production, blastogenesis should not have been suppressed in the present experiment in the presence of a source of exogenous TCGF.

As the follicle of the mare matures towards ovulation, many hormonal changes occur within the follicular fluid (E. D. Watson & P. L. Sertich, unpublished). In particular, there are large increases in concentrations of progesterone, PGF, PGE-2 and 6-keto-PGF-1α between 24 and 36 h after administration of the hCG. High concentrations of progesterone are known to suppress lymphocyte blastogenesis (Low & Hansen, 1988) and PGE-2 suppresses both mitogen- and IL-2-stimulated lymphocyte blastogenesis in cows, ewes (Low & Hansen, 1988), and mares (E. D. Watson, unpublished data). The present study shows that hormones in follicular fluid may be acting to suppress lymphocyte blastogenesis, thereby reducing the immune response of the mare to antigens from the oocyte and the embryo. Treatment of follicular fluid with charcoal, heat, and ether extraction of the fluid slightly reduced its suppressive effect on lymphocyte blastogenesis. Both charcoal treatment and ether extraction would remove prostaglandins and gonadal steroids from the fluid but not proteins. It therefore appears likely that the former substances are contributing to the immunosuppression. By contrast, Fahmi *et al.* (1985) found that the lymphocyte inhibitory factors present in bovine follicular fluid were heat-labile which ruled out the possibility that gonadal steroids were responsible. In our study, the high degree of immunosuppression still present after treatments indicates that the main inhibitory factor(s) remains unknown but may be a protein rather than steroid. In conclusion, follicular fluid from mares possess immunosuppressive properties which may function to inhibit an oviducal immune response to the zona pellucida.

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Papers and Articles

Characteristics of cyclicity in maiden thoroughbred mares in the United Kingdom

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The characteristics of the cyclicity of 12 maiden thoroughbred mares kept in two groups were studied over a total of 58 cycles. On average, oestrus lasted 5·3 days and in 60 per cent of the cycles ovulation occurred in the last two days of oestrus. Oestrus and ovulation tended to be synchronised in each group of mares. The mean diameter of single-ovulating preovulatory follicles on the day before ovulation was 41·5 mm and during the seven days before ovulation they grew 2·5 mm/day. More than one follicle ovulated in 19 (33 per cent) of the cycles (seven double ovulations and 12 dioestrous ovulations). All the oestrous mares and 25 per cent of the mares with dioestrous ovulations had uterine oedema on the day before ovulation.

THERE are many reports of ovarian findings and the characteristics of the oestrous cycles of mares. However, almost all of them have been based on ovarian palpation, hormonal data or on slaughterhouse surveys. The introduction of ultrasound imaging has greatly increased the precision with which events in the reproductive tract of the mare can be recorded, and there are several reports on cyclical ovarian changes detected by ultrasonography in riding-type mares (Ginther 1988, 1990, Ginther and Pierson 1989). Some of the data in these reports, such as the size of the preovulatory follicle, appear to differ from the clinical findings of some practitioners in the United Kingdom. Individual breed and climatic variations can confound such studies on the cyclicity of mares. This paper describes the results of investigations of the cyclical characteristics of a group of young maiden thoroughbred mares in the UK and compares these findings with data from other parts of the world.

Materials and methods

Twelve maiden thoroughbred mares aged three to six years and weighing 450 to 550 kg, were kept in groups of five and seven in adjacent paddocks; they had been kept in these same groups for three months before the start of the study. The mares were given free access to hay and received two feeds of concentrates each day. Blood samples were collected daily between mid-June and mid-September, the serum was removed after centrifugation and stored at –20°C until progesterone concentrations were measured by enzyme immunoassay (Boland and others 1985). The serum progesterone concentrations were used to confirm the findings by palpation. Each day the mares were confined to a shed with solid 4 ft-high gates along one side and group-teased with a stallion;

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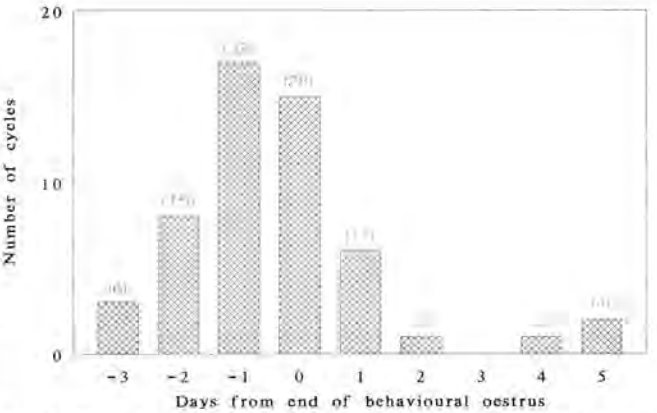

FIG 1: Day of ovulation relative to day of oestrus for 53 oestrous cycles. The figures in brackets are percentages. Day 0 = last day of oestrus

mares known to have follicles more than 30 mm in diameter but not showing signs of oestrus were teased individually. Positive signs of oestrus were defined as squatting, everting the clitoris and urinating, and oestrus was confirmed by the detection of a relaxed cervix, uterine oedema, the presence of a preovulatory follicle and low serum progesterone concentrations. On three days each week the genital tract of each mare was examined by rectal palpation and ultrasonography using a real-time, linear array scanner with a 5 mHz probe (BCF Model 500V; BCF Technology). Any mare in oestrus or with a follicle more than 30 mm in diameter was examined daily until ovulation. The diameter of follicles more than 15 mm was measured and recorded at each examination. For non-spherical follicles, the measurements were made in two dimensions and the average diameter was calculated. Ovulations were classified as occurring during dioestrus if the serum progesterone concentrations were more than 1 ng/ml at the time of ovulation. The texture of the uterine echo was recorded at each examination.

During oestrus the texture is characterised by alternating areas of hyperechogenicity and hypoechogenicity due to the oedematous outer areas of the endometrial folds. During dioestrus the individual endometrial folds are indistinct and as a result the echo is homogeneous (Ginther 1986). The results are given as means ± sd.

Results

A total of 77 ovulations were detected during 58 oestrous periods, and oestrous behaviour was detected in 53 of these periods, giving a silent oestrus rate of 8·6 per cent. Behavioural oestrus lasted 5·3 ± 2·4 days with a range from one to 15 days. Sixty per cent of the ovulations occurred in the last two days of oestrus (Fig 1). The mean interval between ovulations was 21·8 ± 3·5 days with a range from 16 to 35 days. Oestrus and ovulation tended to

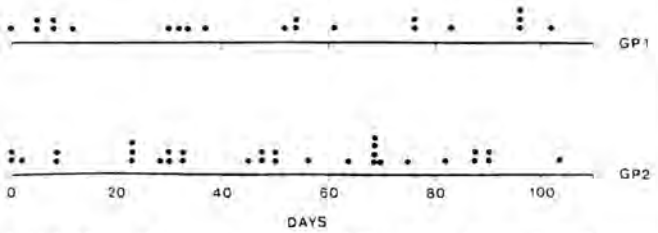

FIG 2: Onset of oestrus in groups of five mares (group 1) and seven mares (group 2)

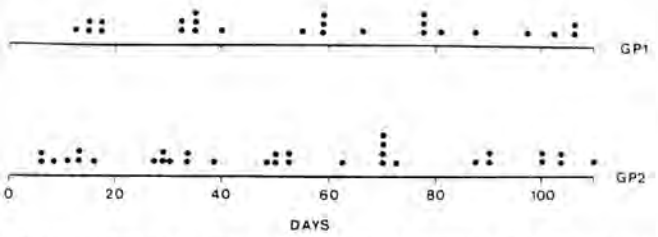

FIG 3: Ovulations in groups of five mares (group 1) and seven mares (group 2). When more than one follicle ovulated at oestrus, only the first follicle was included

be synchronised within, but not between, the two groups (Figs 2 and 3) with most of the mares ovulating within seven days of one another.

The mean diameter of preovulatory oestrous follicles at single ovulations was 41·5 ± 4·9 mm with a range from 31 to 51 mm. During the last seven days before ovulation the preovulatory follicle increased in size at an average rate of 2·5 mm/day (Fig 4). Four single-ovulating oestrus follicles (8 per cent) ovulated at diameters of 31, 31, 34 and 34 mm. Forty-two (55 per cent) of the 77 ovulations occurred on the right ovary and there was no trend for a preferred side of ovulation in individual mares (data not shown). In 19 of the 58 cycles (32·8 per cent) more than one follicle ovulated. On one occasion both follicles ovulated on the same day, and in six cycles there were asynchronous primary ovulations, that is, the follicles ovulated within two to three days of one another during oestrus. The mean diameter of these follicles at double ovulation was 37·8 ± 4·9 mm. In 12 of the 58 cycles dioestrous ovulations occurred three to 23 days (9·4 ± 5·5 days) after the primary ovulation. During the 12 cycles in which a dioestrous ovulation occurred, the mean diameter of the oestrous follicle on the day before ovulation (41·0 ± 5·8 mm) tended to be greater than the diameter of the dioestrous follicle on the day before it ovulated (35·8 ± 8·1 mm) but the difference was not significant. The asynchronous ovulation occurred on the same ovary as the primary ovulation on six of the 12 occasions. Most of the mares ovulated more than one follicle during only one or two oestrous cycles but two mares had four dioestrous ovulations during the period of the study. The interval between two ovulations associated with basal serum progesterone concentrations was significantly lengthened (to 35 days) in only one mare. This mare ovulated three and then 23 days after her primary oestrous ovulation (plasma progesterone = 0) with plasma progesterone concentrations of 4·3 and 4·6 ng/ml, respectively, on the days of ovulation, and went on to ovulate 12 days after her second dioestrous ovulation, with basal concentrations of plasma progesterone.

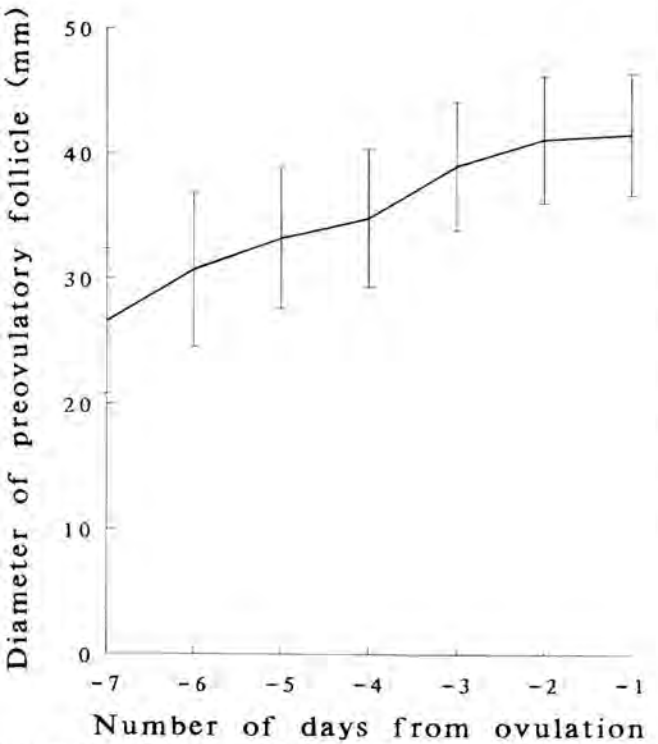

FIG 4: Mean diameter of primary preovulatory follicles during 51 single ovulations. (Day – 7, n = 34; day – 6, n = 38; day – 5, n = 30; day – 4 to day – 1, n = 51)

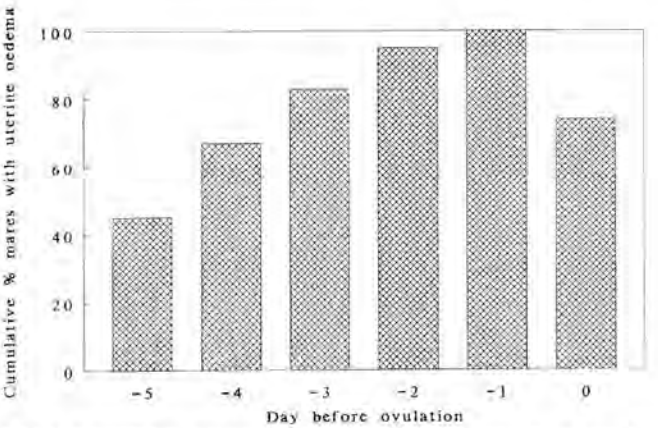

FIG 5: Cumulative percentage of oestrous mares exhibiting uterine oedema before ovulation

Uterine oedema was visible by ultrasonography by five days before ovulation in almost half of the oestrous periods. All the oestrous mares had uterine oedema on the day before ovulation (Fig 5). Uterine oedema was also detected on the day before four of the dioestrous ovulations (33 per cent).

Discussion

Not all the periods of oestrus were accompanied by overt oestrous behaviour, and the incidence of suboestrus or silent oestrus in these mares (8 per cent) was similar to the results from horses in the USA (Cummings 1942, Nelson and others 1985). It appears that silent oestrus may be associated with lower circulating concentrations of oestradiol (Nelson and others 1985) although another study failed to show such a relationship (Munro and others 1979). The length of oestrus, the day of ovulation relative to oestrus and the length of the oestrous cycle agreed with previous reports for horse mares (for review see Ginther 1992).

The synchrony of the oestrous cycles in females kept in groups is a well recognised phenomenon, but to the authors' knowledge has not previously been reported in the mare. It seems unlikely that the group-teasing with the stallion and the exhibition of overt signs of oestrus by other mares in the group induced the synchronisation because the mares in each group appeared to be synchronised before they had been exposed to the stallion. The mares in the two groups, which were separated by a double fence, were not synchronised, suggesting that a direct interaction is needed between mares before their cycles became synchronised.

The mean diameter of the preovulatory follicle on the day before the single ovulations (41·5 mm) was similar to that previously reported in mixed-breed mares (Pierson and Ginther 1985). The preovulatory follicles in these thoroughbred mares were similar in size to the preovulatory follicles of 12 pony mares weighing 230 to 330 kg studied over a total of 22 cycles (40·5 ± 4·6 mm; range 32 to 57 mm; E. D. Watson, unpublished data) demonstrating that ponies do not ovulate smaller follicles than horse mares. The growth rate of the follicles (2·5 mm/day) was slightly less than that reported by Pierson and Ginther (1985) for horse mares (3 mm/day) but this difference may have been due to the period in which these studies were carried out. Follicles appear to grow significantly more quickly in May and June than in July, August and September (Ginther and Pierson 1989).

Only four of the 51 follicles from single ovulations ovulated at diameters less than 35 mm. This observation agrees with those of Ginther (1986) and supports the practice of delaying breeding until a 35 mm preovulatory follicle is present.

More ovulations occurred from the right than from the left ovary in these thoroughbred mares. In a study in the USA 62 per cent of ovulations in maiden mares were from the left ovary (Ginther 1983) and other studies on groups of thoroughbred mares in Australia (Irwin 1966) and New Zealand (Bain and Howey 1975) found either that the ovulation rate from the left ovary was greater or that there was no difference. These results have led to the suggestion that the vascularisation of the left ovary may be greater (Ginther 1992) but the present results do not support the suggestion.

Double ovulations were recorded in seven (12 per cent) of the oestrous cycles. Double ovulations are defined as ovulations which occur not more than two days apart, unless the intervening period is clearly still part of the follicular phase (Ginther 1992). Reliable estimates of multiple ovulations have only recently been made possible by the introduction of ultrasound techniques. In thoroughbred mares the incidence of double ovulations, as determined by palpation, has been reported to be 15 to 25 per cent (for review see Ginther 1986). The frequency of multiple ovulations appears to be related to breed, and thoroughbreds are known to ovulate multiple follicles more frequently than standardbreds, quarter horses or ponies (Ginther 1986). The lower incidence of double ovulations observed in the present study may have been due to an overestimate in the palpation studies reported by Ginther (1986) or to the fact that young mares tend to have a lower double ovulation rate than older mares (Henry and others 1982). In the present study double-ovulating follicles tended to ovulate when they were smaller than single-ovulating follicles. This finding has also been reported in a survey of quarter horse mares (Ginther and Pierson 1989) and has led to the suggestion that it may be important to initiate breeding earlier in mares with two preovulatory follicles if it is intended to breed the mare before ovulation (Ginther 1986).

There was a high frequency of dioestrous ovulations (21 per cent) in agreement with a previous report from California (Hughes and others 1985). On only one occasion was a dioestrous ovulation associated with an extended interovulatory interval (35 days) which indicated that the other secondary corpora lutea were responsive to endogenous prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) at the time of luteolysis. The high incidence of these dioestrous ovulations may explain the apparent failures after the administration of $PGF_{2\alpha}$ during dioestrus, if the corpus luteum resulting from the secondary ovulation is not yet responsive to $PGF_{2\alpha}$ (less than five days old) at the time of treatment. Although Ginther and Pierson (1989) concluded that a spontaneous prolongation of the lifespan of the primary corpus luteum remains to be convincingly demonstrated, and that a prolongation of the luteal phase is more likely to be caused by a late dioestrous ovulation, one of the mares in the present study ovulated in the presence of high concentrations of progesterone 23 days after an oestrous ovulation. This was presumably associated with a failure of the release of $PGF_{2\alpha}$ from the endometrium at the normal time (days 14 to 15) in the oestrous cycle.

It has been reported that some mares repeatedly have multiple ovulations at oestrus (Ginther 1992). Two of the present mares repeatedly ovulated follicles during dioestrus, suggesting that some mares may similarly tend to repeat this type of cycle.

All the mares had uterine oedema on the day before ovulation. The presence of uterine oedema is therefore a valuable diagnostic aid in mares which show only weak signs of oestrus or when mares cannot be teased. However, uterine oedema was also present on the day before ovulation in four of the 12 cases of dioestrous ovulation, which may lead to confusion with oestrous preovulatory follicles if the mares are not teased. Moreover, to complicate the diagnosis of dioestrous ovulations, one of the mares showed transient signs of oestrus before ovulating a follicle during dioestrus. Although mares can conceive at dioestrous ovulations, the chance of introducing an infection leading to endometritis is greatly increased. These findings highlight the importance of evaluating all the components of oestrus, that is, the behavioural, ovarian and uterine structural changes and, if necessary, progesterone concentrations in the blood, in the selection of mares for natural service or artificial insemination. This is particularly relevant for veterinary practitioners who cannot examine a mare daily during oestrus or when the stage of the mare's cycle is unknown before an examination.

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Abstracts

Adenovirus-associated pneumonia and hepatitis in four llamas

THE results of immunofluorescent antibody assays were positive for adenovirus in two llamas aged five and 11 months and in two adult llamas aged three and four years. However, only the young llamas developed hepatic lesions with adenovirus intranuclear inclusion bodies visible by light microscopy. The clinical signs associated with the infection ranged from lethargy and anorexia to progressive respiratory tract infections which were resistant to treatment, and neurological deficits. Mild to moderate fibrinous pleuritis and peritonitis were consistent findings post mortem.

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Research

Secretion of matrix metalloproteinases 2 and 9 and tissue inhibitor of metalloproteinases into follicular fluid during follicle development in equine ovaries

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Extensive tissue remodelling is required in equine ovaries for follicle growth and development and also migration of the follicle to the ovulatory fossa, where ovulation occurs. The mechanisms for these processes are largely unexplored. Matrix metalloproteinases (MMPs) and their endogenous tissue inhibitors (TIMPs) are important for control of breakdown of extracellular matrix during tissue remodelling. The aims of this study were to determine the pattern and sites of secretion of the gelatinases MMP-2 and -9 and TIMPs into follicular fluid during follicle development in mare ovaries. The predominant gelatinase detected in follicular fluid was MMP-2, which was present in similar amounts throughout follicular development, as demonstrated by zymography. MMP-9 was also present in follicular fluid and secretion increased significantly ($P < 0.05$) with development of follicles from < 10 mm to 11–20 mm in diameter. Follicular fluid also contained TIMP-1, TIMP-2, unglycosylated and glycosylated TIMP-3, and TIMP-4, as shown by reverse zymography. The abundance of TIMPs remained largely unchanged during follicle development. MMP-2 and -9 were localized by immunohistochemistry to stromal cells and granulosa and theca cells. TIMP-1, -2, -3 and -4 were present in granulosa and theca cells of the follicle and in stromal cells and also associated with extracellular matrix of the ovarian stromal tissue. The MMPs and TIMPs are likely to be involved in the regulation of the breakdown of extracellular matrix during tissue remodelling for follicle development and migration to the ovulation fossa in mares.

Introduction

The unique structure of the equine ovary, the large eventual size of the preovulatory follicle (sometimes to > 50 mm in diameter), and its migration to the ovulation fossa and subsequent ovulation (Ginther, 1992) require profound tissue remodelling. The processes of development and particularly migration have been largely open to speculative theories suggesting that the large size of the ovulatory follicle results in pressure that forces it along the line of least resistance to the fossa, or by changes in the extracellular matrix that produce a line of least resistance along which migration can take place (Harrison, 1946; Prickett, 1966). The process of migration can be visualized by transrectal ultrasonography. Approximately 85% of preovulatory follicles undergo a profound change in shape from spherical to pear-shaped or conical, pointing towards the ovulatory fossa, coincident with a decrease in follicle turgidity (Pierson and Ginther, 1985). Ovulation is likely to occur due to controlled focal weakening of the follicular wall caused by apoptosis rather than an increase in intrafollicular pressure (Murdoch, 1995). A tear is visualized by ultrasonography in the follicle wall before ovulation, which is thought to be due to breakdown of ovarian stroma (Carnevale *et al.*, 1988). Morphological examination has demonstrated that rupture of the deep layers of ovarian connective tissue is evident in conjunction with disruption of the surface cuboidal and columnar cells of the ovulation fossa (Witherspoon and Talbot, 1970). However, little is known about the breakdown of extracellular matrix during these processes or the enzymes involved in these remodelling events.

The matrix metalloproteinases (MMPs) are a family of key enzymes involved in degradation of extracellular matrix during tissue remodelling. MMPs possess a broad range of substrate specificities, with the capability of breaking down most extracellular matrix components, including all forms of collagen. MMPs are secreted into the extracellular environment as an inactive pro-form and activity requires cleavage of the pro- domain resulting in a conformational change revealing the active site. The MMPs are regulated by

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a family of specific endogenous inhibitors that currently has four members: the tissue inhibitors of matrix metalloproteinases (TIMPs; Woessner, 1991; Hulboy *et al.*, 1997). TIMPs are also secreted by many cell types and bind in a ratio of 1:1 to form non-covalent complexes with a broad specificity to MMPs, thus inhibiting their enzymatic function and regulating remodelling of extracellular matrix.

Members of the MMP and TIMP families are expressed in the ovaries of mice (Hagglund *et al.*, 1999), rats (Bagavandoss, 1998), sheep (Murdoch and McCormick, 1992), cows (Smith *et al.*, 1996) and humans (Duncan *et al.*, 1998; for a review of MMPs in ovarian function see McIntush and Smith, 1998). Song *et al.* (1999) demonstrated that the gelatinases MMP-2 (gelatinase A, 72 kDa type IV collagenase) and MMP-9 (gelatinase B, 92 kDa type IV collagenase) are secreted *in vitro* by stromal cells of equine ovaries. The gelatinases primarily degrade type IV collagen, which is a major constituent of basement membranes. TIMP-1, -2 and -3 are also secreted by equine ovarian stromal cells. However, little is known about the regulation of MMPs and TIMPs in equine ovaries *in vivo*.

The aims of the present study were to examine the secretion of the gelatinases MMP-2, MMP-9 and the TIMPs into follicular fluid during follicle development and to investigate their likely sites of cellular secretion.

Materials and Methods

Collection and preparation of tissue and follicular fluid samples

Ovaries from mature cyclic mares were collected at post mortem or by ovariectomy. The ovariectomy was performed via a colpotomy incision after appropriate sedation and analgesia, as described by Watson and Sertich (1990). Follicles were identified, their diameters were recorded and follicular fluid was collected by needle aspiration.

Follicular wall coloration and morphology of granulosa cells recovered by curettage of the follicular wall were evaluated and oestradiol concentrations in follicular fluid were measured to distinguish follicles as healthy or atretic. The presence or absence of granulosa cell apoptosis was detected after examination for laddering of DNA and staining with ethidium bromide (Pedersen *et al.*, 2000). Validation of the methods used to classify follicles as healthy or atretic was achieved by retrospective histological analysis of 34 follicles 3–54 mm in diameter (Pedersen, 2000).

Follicular fluid samples from healthy growing follicles were selected from four stages of development determined by the follicle diameters: < 10 mm, 11–20 mm, 21–30 mm and 30–45 mm (mares in oestrus) ($n = 2$ mares per group). Fluid samples were also collected from atretic follicles with the same criteria for sizes ⩽ 30 mm in diameter. Furthermore, two samples of fluid were collected from granulosa cell tumours. All follicular fluid samples were dialysed (7.5 kDa molecular mass cut-off; Tube-O-Dialyser, Chemicon International Ltd, Harrow) for 17 h at 23°C against water, which also removes small ions that can disrupt electrophoresis, and the samples were then stored at –20°C before analysis. Sections of healthy growing follicular wall tissue and underlying ovarian tissue (approximately 7 mm^3) were collected and fixed in 10% (v/v) neutral buffered formalin for 24 h, washed in 70% (v/v) alcohol and embedded in paraffin wax.

Radioimmunoassay for oestradiol

Concentrations of oestradiol were measured directly in follicular fluid without extraction. Fluid was diluted in assay buffer from 1:100 to 1:10 000. The assay was performed as described by Glasier *et al.* (1989). Assay sensitivity was 8 pg ml^{-1} and intra- and interassay coefficients of variation were 4.6 and 7.8%, respectively. Displacement curves produced by serial dilutions of follicular fluid and spiking of samples were parallel to the standard curve.

Identification and measurement of MMP-2 and MMP-9 by zymography

Activity of gelatinases (MMP-2 and MMP-9) in follicular fluid was detected using zymography as described by Rawdanowicz *et al.* (1994) with minor modifications (Riley *et al.*, 1999a). Follicular fluid samples and fluids from atretic follicles (1.0–7.5 µl; optimal volume established as 1.5 µl) were separated by SDS-PAGE (7.5% (w/v) gels; Minigel apparatus; Bio Rad, Hemel Hempstead) using gels containing gelatin (1 mg ml^{-1}) in non-reducing conditions. Gels were washed in 2.5% (v/v) Triton X-100 and incubated in digestion buffer (200 mmol NaCl l^{-1}, 50 mmol Tris l^{-1}, 5 mmol $CaCl_2$ l^{-1}, 1 µmol $ZnCl_2$ l^{-1}, 0.02% (v/v) Brij-35, pH 7.6; all chemicals from Sigma Chemical Co, St Louis, MO) for 18 h at 37°C. The gels were stained in staining solution (0.5% (w/v) Coomassie blue R250 in 30% (v/v) methanol–10% (v/v) glacial acetic acid in H_2O) for 3 h at 23°C, and then destained (staining solution without Coomassie blue) to reveal discrete areas in which degradation of gelatin by gelatinases is localized. A sample of human amniotic fluid collected at term during active labour was used as a positive control, which clearly demonstrated the latent forms of MMP-2 and MMP-9 as characterized by Riley *et al.* (1999a).

Identification and measurement of TIMPs by reverse zymography

Detection of TIMP activity was performed by reverse zymography using a commercially available kit (University Technologies Inc., Calgary) as described by Riley *et al.* (1999b). Follicular fluid samples (1.0–7.5 µl; optimal volume established as 3 µl) were separated by PAGE using 12% (w/v) gels containing gelatin (1 mg ml^{-1}) and an MMP preparation (prepared from BHK-21 cells that constitutively express proMMP-2; University Technologies Inc.). Gels were washed (50 mmol Tris l^{-1}, 5 mmol $CaCl_2$ l^{-1}, 2.5% (v/v) Triton X-100) for 2.5 h at 23°C and incubated in

digestion buffer (50 mmol Tris l^{-1}, 5 mmol $CaCl_2$ l^{-1}) for 17 h at 37°C. The gel was counterstained and then destained (as for zymography with staining and destaining buffers) to detect the presence of protein, predominantly the incorporated gelatin. The presence of TIMPs was determined by their discrete inhibition of MMP activity, visualized as a darker band on a lighter background. TIMPs were identified and characterized by comparison with molecular mass markers, with control standards of conditioned medium containing mouse TIMP-1, -2 and -3 expressed by transfected BHK cells (University Technologies Inc.). The same human amniotic fluid control sample used in zymography was again used as a positive control (Riley *et al.*, 1999a,b). When samples were separated by normal PAGE, without substrate or MMP activity incorporated within the gel, no detectable background protein Coomassie blue staining was observed, thus demonstrating the specificity of the TIMP activities.

Localization of MMPs and TIMPs by immunohistochemistry

MMP-1, -2 and -9, and TIMP-1, -2, -3 and -4 were localized in tissue sections (5 μm thickness) mounted on silane-coated slides using immunohistochemical techniques as described by Riley *et al.* (1999b, 2000). Sections were dewaxed, rehydrated and endogenous peroxidase activity was inhibited by incubation in H_2O_2 (3% (v/v) solution in H_2O for 20 min). Sections were washed; a blocking solution was applied (normal goat serum; 30 min) followed by the primary antibody for 17 h at 4°C. The following primary antibodies were used: MMP-2 (mouse monoclonal: 10–20 μg ml^{-1} working concentration; Calbiochem, Nottingham); MMP-9 (rabbit polyclonal: 0.1 μg ml^{-1}; Insight Biotechnology, Wembley); TIMPs-1, -2, -3 and -4 (all rabbit polyclonal: 0.1–1.0 μg ml^{-1}; Triple Point Biologics, Forest Grove, OR). The primary antibody was detected using a biotinylated mouse anti-horse or goat anti-rabbit antibody as appropriate. It was visualized using an avidin–peroxidase complex with 3,3′-diaminobenzidine as chromagen (appears brown) according to the manufacturer's instructions (Vector Laboratories, Burlingame, CA). Sections were counterstained with Harris's haematoxylin, dehydrated, mounted and visualized by light microscopy. Negative controls were performed in parallel in which the primary antibody was omitted.

Data and statistical analysis

MMP and TIMP activities as identified by zymography and reverse zymography were measured by densitometric analysis. These techniques can discriminate among different MMP and TIMP forms by molecular mass and their relative activities. Relative activity densities (expressed as arbitrary absorbance readings) were derived from zymography gels by comparison with parallel background readings of equal area and calculated using dedicated software (Quantity One; Bio-Rad). Densitometric readings were compared only with the other gel examined under exactly the same conditions (this being the same electrophoresis run and identical buffers, stains and incubation periods) as described by Riley *et al.* (1999b, 2000). Results were analysed using Student's *t* test to compare between oestradiol concentrations and also densitometric values reflecting activities of different MMPs in samples collected from follicles of the same size, or ANOVA with a post hoc Tukey's test to compare activities of an MMP between follicles of different sizes.

Results

Assessment of follicles

All the follicles used in these studies were identified and classified initially as healthy or atretic according to macroscopic criteria at the time of dissection. This classification was confirmed by retrospective analysis including histological examination (data not shown). The follicles judged by histological criteria to be atretic (Kenney *et al.*, 1979) all had high degrees of apoptosis detected by DNA laddering, whereas all follicles judged by histological examination to be healthy, had no or very low levels of DNA laddering. Furthermore, oestradiol concentrations in fluid collected from small (< 10 mm in diameter) follicles (44.7 ± 31.7 ng ml^{-1}) were significantly ($P < 0.01$) lower than in the largest (31–45 mm in diameter) follicles (1021.2 ± 286.8 ng ml^{-1}). Similarly, as a further marker for atresia, oestradiol concentrations in atretic follicles of all sizes (35.8 ± 16.0 ng ml^{-1}) were lower than in healthy follicles, irrespective of size.

Activity of MMP-2 and MMP-9 in follicular fluid during follicular development

In follicular fluid, the predominant gelatinase activity was present at 72 kDa, corresponding to the latent form of MMP-2, as demonstrated by zymography (Fig. 1). This finding was consistent, regardless of the stage of development of the follicle. Detection of active MMP-2 (66 kDa) was less consistent and was detected in only four of 20 follicular fluid samples with no correlation with the stage of follicle development. Gelatinase activity was also detectable at 92 kDa, corresponding to latent MMP-9 with low amounts secreted into follicular fluid of follicles < 10 mm in diameter. The abundance of MMP-9 increased significantly ($P < 0.05$) in the 11–20 mm group when compared with the 0–10 mm group, and then remained at this higher level in the larger follicles. There were lower amounts of the latent forms of MMP-2 and -9 in fluid collected from atretic follicles (Fig. 2), which were remarkably consistent in samples from follicles of all sizes, although there was an increase in the amount of the active form of MMP-2 in atretic follicles. Fluid samples collected from two granulosa cell tumours also contained both MMP-2 and MMP-9 (Fig. 2).

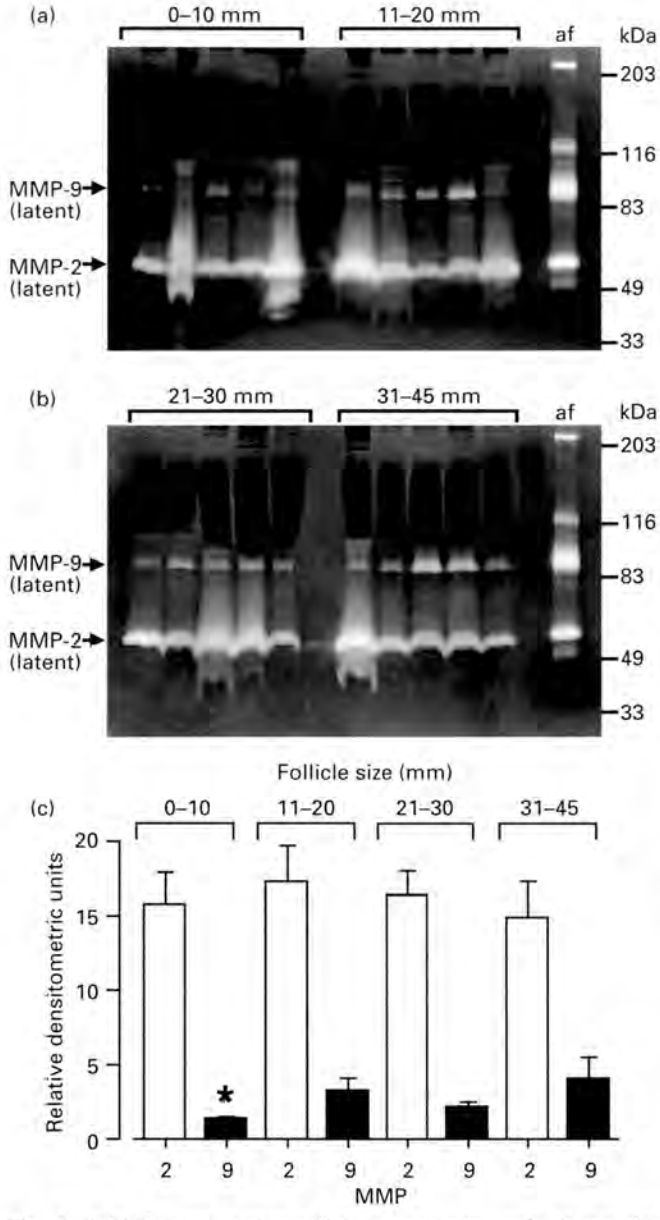

Fig. 1. (a,b) Representative gelatin zymography gels of samples of equine follicular fluid (1.5 µl analysed) from follicles in the size ranges 0–10 mm, 11–20 mm, 21–30 mm and 31–45 mm diameter. af: human amniotic fluid control sample. (c) Relative abundance (arbitrary absorbance readings; mean ± SEM) of matrix metalloproteinase 2 (MMP-2; □) and -9 (■) determined by densitometric analysis. MMP-9 but not MMP-2 activity increased significantly from follicles 0–10 mm in diameter to follicles 21–45 mm in diameter. *Significantly ($P < 0.05$) lower MMP-9 activity compared with the larger follicle sizes.

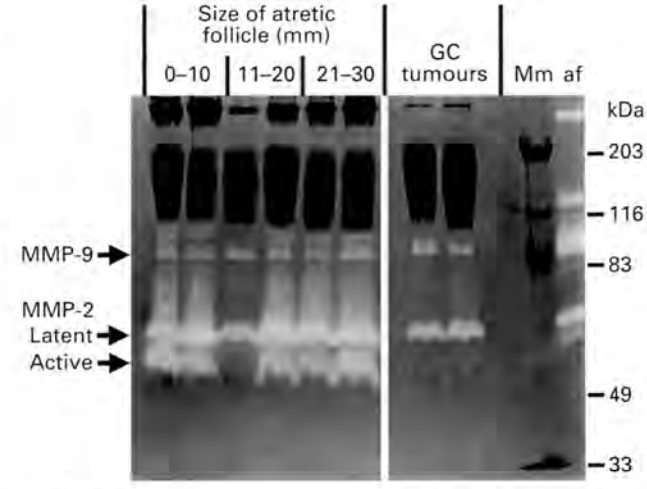

Fig. 2. Gelatin zymography gel of samples of equine follicular fluid (5.0 µl analysed) collected from atretic follicles of a range of different sizes. Samples of fluid collected from granulosa cell ovarian tumours (GC tumours) from two mares are also demonstrated. The predominant gelatinase activity detected is matrix metalloproteinase 2 (MMP-2), both latent and active forms. Mm: molecular mass markers. af: human amniotic fluid control sample.

Activity of TIMPs in follicular fluid during follicular development

A broad range of TIMP activities was detected as three bands by reverse zymography at 27–30 kDa, corresponding to the molecular mass of TIMP-1, glycosylated TIMP-3 and TIMP-4, at 24 kDa corresponding to unglycosylated TIMP-3 and at 21 kDa corresponding to TIMP-2 (Fig. 3). Reverse zymography is unable to distinguish precisely among the TIMP isoforms of 27–30 kDa molecular masses. There were no significant differences in the relative abundance of the different TIMPs during follicle development.

Cellular localization of MMP-2 and -9, and TIMP-1, -2 and -4 in the follicle and ovary

Specific positive immunostaining for MMP-9 was localized consistently in theca and granulosa cell layers in follicular wall samples at stages of follicle development where follicles were > 5 mm in diameter (Fig. 4a). Findings were less consistent in follicles < 5 mm in diameter, with some positive staining in theca and granulosa layers. MMP-9 was also localized in perivascular cells and weakly in the ovarian stroma, in fibroblast cells and on extracellular matrix. MMP-2 was present consistently in ovarian stroma in all samples, and in follicle tissue taken from follicles > 5 mm in diameter the theca and granulosa layers were consistently negative (data not shown). MMP-1 was localized predominantly in theca cells of the follicle wall only. TIMP-1 (Fig. 4c), TIMP-2 (Fig. 4d) and TIMP-4 (Fig. 4f) were localized to both theca and granulosa layers at all stages of development. TIMP-3 was localized in theca cells, with little present in granulosa cells (Fig. 4e). Furthermore, all four members of the TIMP family were localized weakly to extracellular matrix and fibroblast cells in the stromal region (Fig. 4b,d,e,f), with TIMP-1, -2 and -3 being localized strongly in cells with a macrophage-like morphology (Fig. 4b,d). TIMP-2 (Fig. 4d) and TIMP-3 were also present in the perivascular and smooth muscle cells surrounding the vasculature. Non-

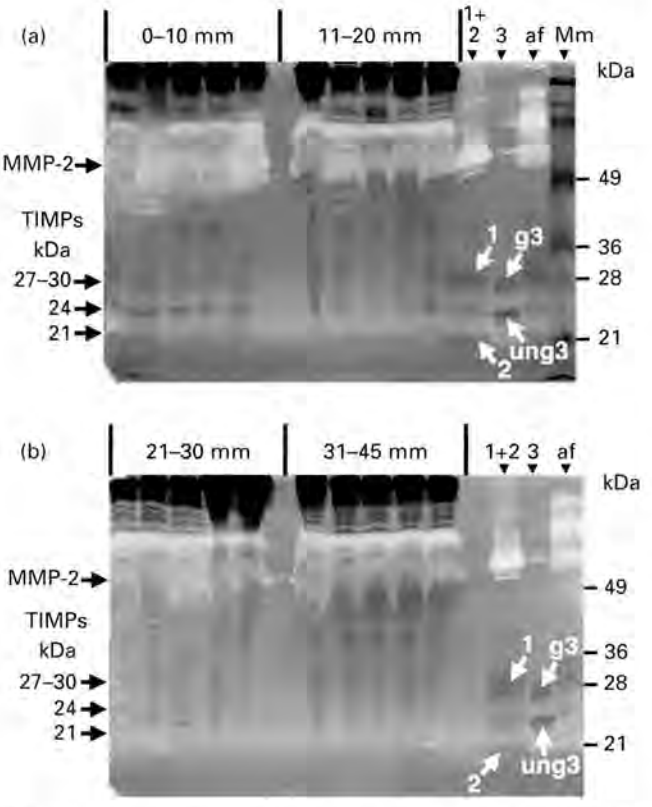

Fig. 3. Representative reverse zymography gel demonstrating tissue inhibitor of matrix metalloproteinase (TIMP) activities in mare follicular fluid samples from follicles of size ranges (a) 0–10 mm and 11–20 mm and (b) 21–30 mm and 31–45 mm in diameter. TIMP activity is visualized as three darker bands of 27–30 kDa (corresponding to the molecular masses of TIMP-1, glycosylated TIMP-3 and TIMP-4), 24 kDa (corresponding to unglycosylated TIMP-3) and 21 kDa (corresponding to TIMP-2). These are compared with controls (identified by arrows) for TIMP-1 and -2 (1+2), and TIMP-3 (3; two bands corresponding to the unglycosylated (24 kDa; ung3) and glycosylated (27 kDa; g3) isoforms) and to TIMPs in human amniotic fluid collected at term, used as a positive control (af). Mm: molecular mass markers.

specific staining was not observed in any negative control sections (for representative section see Fig. 4g).

Discussion

In the present study the presence of MMP-2 and -9 in equine follicular fluid at all stages of development has been demonstrated by gelatin substrate zymography. The most abundant gelatinase activity was due to the latent form of MMP-2 (72 kDa), which was secreted in similar amounts in all follicle sizes from < 10 to 45 mm in diameter. Activity for MMP-9, although less than that of MMP-2, was also present. The concentrations of MMP-9 in follicular fluid increased significantly from the smallest follicle size (< 10 mm in diameter) to follicles 11–20 mm in diameter. A broad range of members of the TIMP family was also present in follicular fluid. These TIMP concentrations were maintained at similar values throughout follicle development. Localization of MMPs and all members of the TIMP family by immunohistochemistry revealed a complex cellular and spatial distribution. MMPs and TIMPs were localized in cells of the follicle, in the underlying stroma in several cell types including stromal fibroblasts and macrophage-like cells, cells associated with the vasculature, and also with extracellular matrix. MMP-2 was the most abundant gelatinase in fluid collected from atretic follicles and was present in both its active and latent forms, although the overall MMP-2 activity was much lower than in healthy follicles.

The presence of MMP-2 and -9 in follicular fluid, which have substrate specificities that include the basement membrane constituent collagen IV, indicates that they are likely to be required for tissue remodelling during follicle growth and development. Furthermore, the localization and secretion (Song *et al.*, 1999) of these MMPs in cells of the ovarian stroma indicate a possible role in the migration of the follicle to the ovulation fossa. The corresponding increase in abundance of MMP-9 in larger follicles indicates strongly that MMP-9 is involved in these ovulatory processes. Ovulation is probably a result of controlled enzymatic degradation and loss of collagen in the follicle wall (Espey, 1994; Luck and Zhao, 1995), combined with local apoptosis (Murdoch, 1995) and not due to an increase of intrafollicular pressure. Collagenolytic enzymes are thought to play a vital role in ovulation in other species, including rats and macaques (Reich *et al.*, 1985; Hirsch *et al.*, 1993; Espey, 1994; Chaffin and Stouffer, 1999). This includes the fibrillar collagenases such as MMP-1, which break down the fibrillar collagen forms that confer much of the structural integrity to the ovarian stroma. From our studies, the possibility that there is a local and acute increase in MMPs with a concomitant decrease in TIMPs around the time of ovulation cannot be eliminated. Profound tissue remodelling occurs during the 6–12 h before ovulation as the follicle alters its shape from spherical to conical, directed towards the ovulatory fossa. Future studies will examine further the spatial and temporal remodelling at this time. Another candidate for the regulation of ovulation may be stimulation of MMP-2 activation, which is present in follicular fluid at all stages. MMP-2 can bind selectively to integrin $\alpha V\beta 3$ (Brooks *et al.*, 1996) to confer a specific localization and can be activated subsequently by membrane bound MMPs, including MMP-14, which are regulated by ovarian steroids (Seiki, 1999; Zhang *et al.*, 2000), thus providing both spatial and hormone-regulated temporal control.

In the present study, a significant increase in MMP-9 concentrations in follicular fluid was observed from small (< 10 mm in diameter) to medium-sized (> 11 mm in diameter) follicles. Little is known about follicular gonadotrophin sensitivity in mares, but LH binding and mRNA encoding LH receptors increase in similar size ranges of follicles (> 10 mm in diameter) with associated increases in

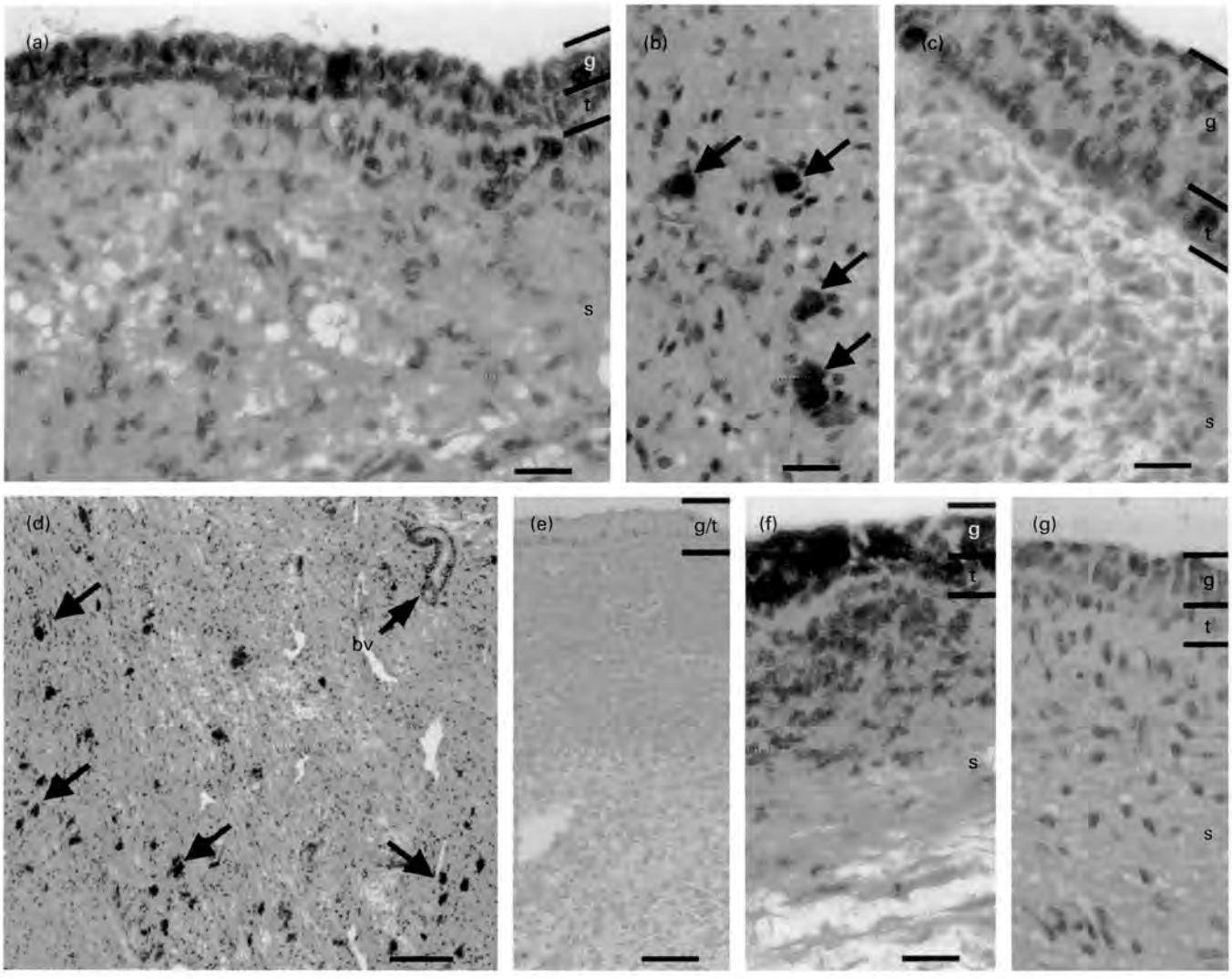

Fig. 4. Photomicrographs of representative tissue sections of equine follicles showing the localization of matrix metalloproteinase 9 (MMP-9) and tissue inhibitor of matrix metalloproteinase 1 (TIMP-1), TIMP-2 and TIMP-4 by immunohistochemistry. (a) MMP-9 in granulosa and theca cells of the wall of a follicle 7 mm in diameter, and in underlying stromal tissue. (b) TIMP-1 in the stromal tissue in cells with macrophage-like morphology (marked by arrows). (c) TIMP-1 in granulosa and theca cells of the wall of a follicle 23 mm in diameter. (d) TIMP-2 in the ovarian stroma showing diffuse positive staining in fibroblast cells and extracellular matrix with some intense immunostaining in cells with macrophage-like morphology (marked by arrows), and in perivascular cells surrounding a blood vessel. (e) TIMP-3 predominantly in theca cells and stromal cells and associated with matrix underlying the theca layer. (f) TIMP-4 predominantly in the granulosa and theca cells of the wall of a follicle 23 mm in diameter, and in underlying stromal tissue. (g) Representative negative control section demonstrating no non-specific staining. bv: blood vessel; g: granulosa cell layer; g/t: granulosa and theca cell layers; s: stromal tissues; t: theca cell layer. Scale bars represent (a, b, c, f and g) 20 μm, (e) 50 μm and (d) 100 μm.

oestrogen concentrations in follicular fluid (Fay and Douglas, 1987; Lawler *et al.*, 1998; Goudet *et al.*, 1999). This finding reflects an increase in aromatase activity, as androgen concentrations are high in small follicles (Fay and Douglas, 1987). Aromatase is expressed only in large (> 10 mm in diameter) healthy follicles, but is absent from atretic follicles in mares (Watson and Thomson, 1996; Goudet *et al.*, 1999).

In mares, as in other species, the presence of oestrogen in follicular fluid is thought to be anti-atretogenic (Harman *et al.*, 1975; Kenney *et al.*, 1979). Androgens do not appear to be associated with atresia in mares (Kenney *et al.*, 1979; Fay and Douglas, 1987). In the present study, follicular fluid oestrogen concentrations were higher in the larger follicles classed as viable compared with follicles classed as atretic. In previous studies, there were no large differences in oestradiol concentrations in follicles > 15 mm in diameter, other than in those follicles classed as preovulatory (Fay and Douglas, 1987). Oestrogens may have a role in stimulating MMP-9 secretion in the larger (> 10 mm in diameter)

follicles. There were no differences in concentrations of TIMPs in follicular fluid throughout follicle development, indicating that they may be expressed constitutively.

It is becoming clear that MMPs and TIMPs have other actions on cell functions besides their role in matrix degradation. MMPs may alter local concentrations of growth factors by releasing growth factors bound to extracellular matrix during breakdown, by cleavage of pro-hormones, for example in the activation of tumour necrosis factor α (Gearing *et al.*, 1995), or by degradation to inactive forms (Hulboy *et al.*, 1997). Furthermore, interactions between the cell and its extracellular matrix control many functions, including the cell cycle and apoptosis, protein synthesis and invasive properties. TIMPs may also have other roles in controlling aspects of follicle development and function. TIMP-1 regulates the cell cycle and differentiation in other cell systems (Corcoran and Stetler-Stevenson, 1996), although it is not known whether this is mediated directly or by interactions with the extracellular matrix. Furthermore, TIMP-1 may be involved in the control of steroidogenesis, as in the testis a complex of TIMP-1 and procathepsin-L stimulates steroidogenesis (Boujrad *et al.*, 1995). It is interesting to speculate whether the wide variety of other effects of MMPs and TIMPs mediated through cell–matrix interactions play a role in equine follicle development, selection and cellular function.

There are several possible cellular sites of secretion of MMPs and TIMPs in equine ovaries. The likely cellular sources of secretion into the follicular fluid are the granulosa, theca and interstitial cells, where MMP-2 and -9, and TIMPs, are localized by immunohistochemistry. However, this does not preclude these cells from taking up these secreted factors. These cell types have been demonstrated to express the appropriate mRNAs both *in vitro* and *in vivo* in mice, rats, cows and women, and to contain the protein and secrete these MMPs and TIMPs (Inderdeo *et al.*, 1996; Bagavandoss, 1998; Duncan *et al.*, 1998; McIntush and Smith, 1998; McCaffery *et al.*, 2000). In mares, Song *et al.* (1999) demonstrated that equine ovarian stromal fibroblasts secrete MMP-2 and -9 and TIMP-1, -2 and -3, as confirmed by the results of the present study. This finding is also consistent with the secretion of MMPs and TIMPs by fibroblasts derived from other tissue sources (see Hulboy *et al.*, 1997; Nagase and Woessner, 1999). Within the ovarian stroma, we have also identified a population of cells with macrophage-like morphology that contain immunoreactivity to TIMPs and MMPs. These cells may be recruited at appropriate stages for local control of tissue remodelling, or may be resident and regulated within the ovarian stroma. MMP-2 and -9 are also secreted into cysts in granulosa cell tumours. MMPs are likely to be required for tissue remodelling during the formation of these large masses (Watson, 1999) and may be stimulated by local steroid production by the tumour.

In conclusion, the major gelatinase activity present continuously in follicular fluid is MMP-2, whereas MMP-9 activity, although in lower abundance, increases in follicular fluid during early development. A broad range of TIMPs was present in follicular fluid at all stages of development. MMP-2 and -9, and TIMPs are likely to play a role in breakdown in extracellular matrix during follicle growth and development, in follicle migration and in ovulation and in controlling other cell functions, including the cell cycle. In future studies, MMP and TIMP expression will be examined in ovarian tissue from mares in the period immediately preceding ovulation to characterize better the temporal changes involved. The roles and cellular control mechanisms of other members of the MMP family in follicle development in equine ovaries are currently under investigation.

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OVARIAN FOLLICULAR RESPONSE OF MARES TO GnRH AGONIST (LEUPROLIDE ACETATE) TREATMENT AFTER PITUITARY SUPPRESSION

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ABSTRACT

Follicular growth and ovulation were monitored in 18 horse mares during a control cycle and during a cycle in which the mares received a GnRH agonist, leuprolide acetate (LA; 200 or 400 μg), twice daily until ovulation. Prior to both of these cycles, follicular growth was suppressed using a 10-day estrogen-progesterone treatment regimen, with prostaglandin F-2α (10 mg) administered on Day 10. Four of the mares treated with LA remained anovulatory for at least 3 weeks after the end of treatment and were excluded from statistical analysis. The dosage of LA did not affect response. Treatment with LA significantly (P=0.0375) increased the percentage of large follicles per ovulation (i.e., follicles greater than 30 mm in diameter on the day on which the largest follicle reached 35 mm) and also increased (P=0.0539) the diameter of the second largest follicle. However LA did not significantly alter the number of ovulations. Mean daily concentrations of luteinizing hormone (LH) were not significantly different during treatment and control cycles. The LH in blood samples collected repeatedly on Day 19 after the start of estrogen-progesterone treatment did not show a difference in frequency or amplitude of pulses between treatment and control cycles. Mares were artificially inseminated during estrus and the embryos were recovered. Fewer embryos were recovered per ovulation from mares after treatment with LA (26%) than during the control cycle (64%). Results indicate that treatment with LA either suppressed follicular activity or induced multiple follicular growth.

Key words: superovulation, leuprolide acetate, mare

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INTRODUCTION

The technique of embryo transfer is widely used in the horse industry. Further advances will be limited, however, until a reliable method of inducing superovulation in mares is available commercially. Mares are seasonally polyestrous, and, within one estrous cycle, only about 16% of mares ovulate more than one follicle (1). Attempts to induce the growth of multiple preovulatory follicles in the seasonally anestrous mare have been successful using once- or twice-daily injections of an equine pituitary preparation (2,3) or pulsatile administration of gonadotropin releasing hormone (GnRH) via an in-dwelling infusion catheter (4). However, these methods of treatment are expensive, labor intensive, seasonally restrictive, and, in the case of equine pituitary extract, of limited availability. An alternative approach to pulsatile infusion of GnRH has become available with the introduction of long-acting GnRH agonists. Twice-daily injections of a GnRH agonist,[a] induced follicle growth and ovulation in seasonally anestrous mares (5). Another study which also used twice-daily injections of a GnRH agonist reported an increased multiple ovulation rate in seasonally anestrous mares (6).

Attempts to induce growth of multiple follicles in cyclic mares have been less successful and until recently have been limited to administration of equine pituitary extract during mid- to late-diestrus (7-9). Infusion of GnRH in cycling mares has not been shown to increase the number of ovulating follicles (10). Successful treatment appears to be related to follicular activity at the beginning of the treatment regimen. To obtain the growth of multiple follicles, the diameter of follicles on the mare ovary at the start of treatment must be less than 30 mm (11). Therefore, it seems that superovulation regimens should incorporate a period of pituitary suppression prior to commencement of superovulatory therapy.

Leuprolide acetate[b] a long-acting GnRH agonist which is similar pharmacologically to Leutrelin, is now commercially available. Both leuprolide acetate and Leutrelin have D amino acids substituted at Position 6 and prolene substituted at Position 9. These alterations in structure result in a 144-fold increase in potency over the endogenous hormone because of increased binding affinity at the receptor sites (12). It is possible that GnRH therapy in cyclic mares has been unsuccessful due to the negative feedback of hormones (estradiol and/or inhibin-like substances) secreted by the dominant follicle or by the other growing follicles. In the present experiment, we monitored stimulation of follicular growth and development by leuprolide acetate after a period of suppression of follicular development using an estradiol-17β (estrogen) + progesterone treatment regimen.

MATERIALS AND METHODS

Eighteen light horse mares, between 3 and 11 years of age and weighing 450 to 550 kg, were used in the study. The experiment started in April when all the mares were considered to have commenced cyclic ovarian activity based on per rectum palpation and ultrasonographic examination of the genital tract, and ended in June. Endometrial biopsies collected later in the summer from these 18 mares showed 17 of them to be in Category I and one mare to be in Category IIA, according to the criteria of Kenney and Doig (13). All the mares received daily injections (2ml i.m.) of 150 mg progesterone + 10 mg estrogen in corn oil for 10 days to suppress follicular development (14). On Day

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[a] Leutrelin, Wyeth Pharmaceuticals, Philadelphia, PA.
[b] Lupron, TAP Pharmaceuticals, Chicago, IL.

10, the mares received a single injection of 10 mg prostaglandin F-2alpha.[c] During the first cycle under study, the mares received no further treatment. During the second cycle under study, the mares were divided into two groups balanced for breed. Each group received 200 or 400 µg leuprolide acetate in 2 ml, i.m. of sterile water twice daily from the day after termination of progesterone + estrogen treatment until ovulation. If ovulation did not occur within 21 days of the start of leuprolide acetate treatment, treatment was discontinued.

The genital tract was examined by per rectum palpation and ultrasonography on Day 10 and then daily from Day 16 until ovulation. When the largest follicle reached 35 mm, the mares received 2000 IU i.v. hCG[d]. On the day a 30-mm follicle was detected, mares were bred artificially using extended semen from two fertile stallions. Thereafter, the mares were inseminated every second day until the day of ovulation. Embryos were recovered nonsurgically 8 days after ovulation using a technique described previously (15) with the modification that the flushing fluid was left in the uterus for 3 minutes prior to recovery, as described by Hinrichs (16).

Heparinized blood samples were collected daily by jugular venipuncture from Day 10 until 8 days after ovulation. Blood was centrifuged at 2000 g for 10 minutes at 4°C. Plasma was stored at -20°C until assayed for progesterone concentrations. Plasma collected from Day 10 until the day on which hCG was administered was also assayed for luteinizing hormone (LH). Blood samples were collected on Day 19 at 20-minute intervals for 4 hours. These samples were assayed for LH concentrations.

Luteinizing hormone was measured in the plasma by the method of Thompson et al. (17). A purified equine LH standard (E98A) was supplied by Dr. H. Papkoff, University of California, while the ovine LH obtained from Dr. L.E. Reichert, Jr., Albany Medical College, was radioiodinated and used as the tracer. The antiserum for the LH assay was raised in rabbits against equine chorionic gonadotropin (eCG) and was supplied by Dr. D.L. Thompson, Jr., Louisiana State University. Cross reactivities of this antiserum with highly purified equine follicle stimulating hormone (FSH) and thyroid stimulating hormone (TSH) were 2.4 and 3.8%, respectively, when compared with equine LH. Sensitivity of the assay was 2.5 ng/ml plasma using 100 µl samples. Addition of LH standards to plasma yielded a regression equation of $y=1.0x + 0.01$ and a coefficient of correlation = 0.99. Intra- and inter-assay coefficients of variation were 6.7% and 11.4%, respectively.

The response of mares to treatment was analyzed using analysis of variance for repeated measures. Statistical analysis of follicle numbers was restricted to the percentage of follicles per ovulation, since this was dependent upon number of ovulations. Control and treated values and dose effects were compared simultaneously. The LH pulse frequency and amplitude in the blood samples collected at 20-minute intervals were defined and analyzed as described by Fitzgerald et al. (18). For statistical analysis, hormone concentrations below the limit of detection of the assays were considered to be zero. All means are quoted ± SEM.

RESULTS

During the control cycle, all of the mares developed large follicles which subsequently ovulated. However, during the leuprolide acetate treatment cycle, four

[c] Lutalyse, The Upjohn Co., Kalamazoo, MI.
[d] Follutein, Solvay Veterinary Inc., Princeton, NJ.

mares (one receiving 200 μg of leuprolide acetate and three receiving 400 μg of leuprolide acetate) did not ovulate until 30, 31, 34 and 34 days after the conclusion of progesterone + estrogen treatment. The ultrasonographic findings were confirmed by measurement of basal concentrations of plasma progesterone during the period prior to ovulation. These four mares were excluded from statistical analysis during both their control and treatment cycles.

There was no significant difference between treatment and control cycles in the interval between the end of progesterone + estrogen treatment and detection of a 35-mm follicle (Table 1). Ovulation of all follicles occurred between 24 and 48 hours after administration of hCG during the control cycle and between 24 and 96 hours after injection of hCG during the treatment cycle.

The diameter of the second largest follicle on the day the preovulatory follicle reached 35 mm was greater (P=0.0539) during the leuprolide acetate treatment cycle (29.0 ± 1.2 mm) than during the control cycle (22.3 ± 2.0 mm). There was no difference between dosages of leuprolide acetate. There was a significant increase in the percentage of large follicles (i.e., greater than 30 mm when the largest follicle reached 35 mm) per ovulation with leuprolide acetate treatment (P=0.0375), but there was no difference between dosages of leuprolide acetate. There was no effect of treatment or of dosage of leuprolide acetate on the number of ovulations (Table 2).

Concentrations of LH in the blood samples collected once daily were not affected by treatment or dosage of leuprolide acetate (Figure 1). Mean concentrations of LH in plasma collected at 20-minute intervals on Day 19 did not differ significantly in treatment versus control cycles (data not shown). Pulse frequency and amplitude in the 4-hour sampling period did not vary significantly between the control (1.4 ± 0.25 pulses/4 hours; 1.3 ± 0.17 ng/ml) and treatment (0.9 ± 0.22 pulses/4 hours; 1.1 ± 0.13 ng/ml) cycles. In the four mares that remained anovulatory during the treatment cycle, mean LH concentrations, pulse frequency and amplitude in the samples collected at 20-minute intervals for 4 hours on Day 19 were not significantly different between the control and treatment cycles (data not shown).

Two mares during the treatment cycle and two other mares during the control cycle developed endometritis after breeding. These mares were not included in the embryo recovery data. Morphology and size of all embryos collected were considered to be within normal limits for 8-day horse embryos. The number of embryos recovered per flush tended to be lower in leuprolide acetate-treated mares (Table 3) than in the control group and when the yield of embryos was related to number of ovulations, markedly fewer embryos were recovered from leuprolide acetate-treated than from control mares.

Table 1. Interval from end of progesterone and estrogen therapy until a 35-mm follicle or ovulation was detected on the ovaries of 14 mares.

Duration	Control cycle	Treatment cycle
End of progesterone and estrogen therapy to 35-mm follicle(days)	11.2 ± 0.6	10.9 ± 0.7
End of progesterone and estrogen therapy to ovulation (days)	13.1 ± 0.7	13.1 ± 0.7

There was no significant affect of treatment or dosage.

Table 2. Follicular growth and ovulations in mares during control cycles and during treatment with leuprolide acetate

	Control cycle	Treatment cycle
No. of mares	14	14
No. of follicles >30 mm on the day the largest follicle reached 35 mm	1.21 ± 0.11	1.86 ± 0.23
No. of ovulations/mare	1.29 ± 0.13	1.57 ± 0.23
No. of large follicles/ovulation	0.96 ± 0.04	1.25 ± 0.11*

* P = 0.0375.

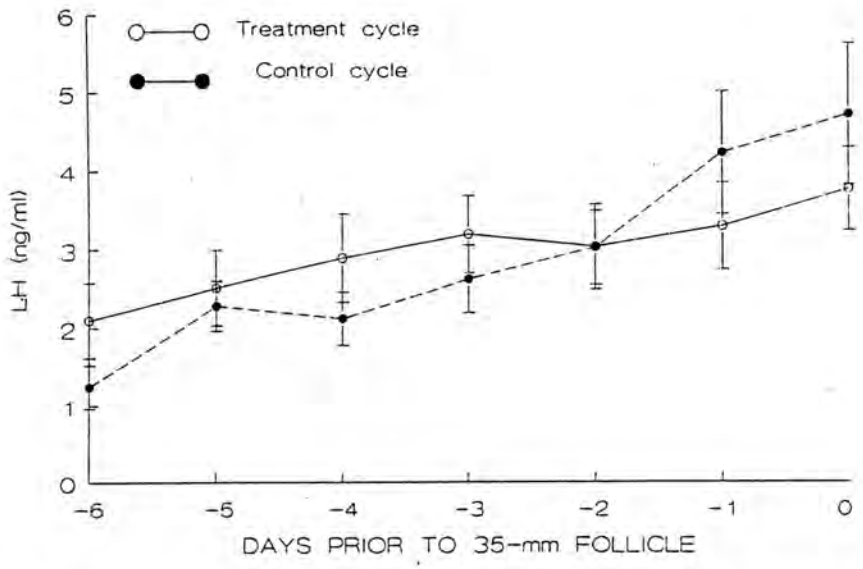

Figure 1. Concentrations (mean ± SEM) of LH in daily plasma samples collected in the 6 days preceding detection of a 35-mm follicle on the ovaries from mares during control cycles and during treatment with leuprolide acetate. Because the dosage of leuprolide acetate had no effect on LH concentrations the treatment groups were combined.

Table 3. Effect of treatment with leuprolide acetate on embryo recovery

Treatment	No. of Ovulations[a] 1	2	3	No. of embryos recovered	No. of ovulations yielding embryos
Control	6/10	3/2	—	9/12(75%)	9/14(64%)
Leuprolide acetate	3/8	1/1	1/3	5/12(42%)	5/19(26%)

[a]Figures denote the number of embryos recovered/number of mares flushed.

DISCUSSION

Twice-daily injections of leuprolide acetate after combined progesterone + estrogen therapy increased the number and size of follicles in mares, but they were also associated with prolonged periods of anovulation in others. In a previous study (P.L.Sertich, unpublished data) in which the treatment regimen was the same as that of the control cycle in the present study, the average number of follicles >30 mm per mare when the largest follicle reached 35 mm in diameter was 1.3 in each of March (n=9), April (n=62) and May (n=14) and 1.4 in June (n=15). It appears, therefore, that the month of treatment probably had little effect on follicular growth in the present study and that the increased follicular growth in treated mares could be attributed to treatment with leuprolide acetate without significant seasonal effects. The period to ovulation after similar progesterone + estrogen treatment regimens has been reported as 9 to 13 days after the last injection (19). In one study, the mean interval from last injection until appearance of a 35-mm follicle was 11.8 $\pm$ 0.6 days (11), which is similar to the results in the present study. It would appear, therefore, that those four leuprolide treated-treated mares which did not have follicles larger than 30 mm and which did not ovulate for at least 30 days after the last injection were outside normal limits for this treatment regimen. It was recently reported that chronic administration of high dosages of GnRH agonist suppressed serum LH concentrations in ovariectomized mares (20), suggesting desensitization of the pituitary. However, the four mares in our study showed no evidence of decreased concentrations of LH during their treatment cycle. Similarly, another study showed the pituitary of anoestrous mares did not become refractory to chronic administration of GnRH after 10 to 16 days (4). It is possible that the GnRH agonist used in the present study caused down-regulation of LH receptors at the ovarian level. Three of the four anovulatory mares were in the group being treated with the higher dosage of leuprolide acetate; perhaps it would have been advantageous to have used a lower dosage. In agreement with a previous report (6), the dosages of GnRH agonist that we chose did not alter follicle response in the mares that went on to ovulate during treatment. In the present study, no differences in daily plasma LH concentrations were detected between single and multiple ovulating mares. These findings are in accordance with those of other investigators (4,21). It is probable that the stimulating effect on follicular recruitment that appeared to be mediated in some mares in the present study by leuprolide acetate was caused by increased secretion of FSH. Follicle stimulating hormone is known to be important in stimulation of follicles to develop to the preovulatory stage. Induction of multiple preovulatory follicular development in women is thought to depend on stimulation with FSH rather than LH (22), and in mares

a significant correlation has been reported between FSH concentrations and the number of ovulations (4).

Although in some mares leuprolide acetate treatment was successful in stimulating a higher number of large follicles than during the control cycle, some of these follicles failed to ovulate. This problem has been encountered by other researchers attempting to induce superovulation in mares using pituitary extract (7,23) or FSH (24). A greater proportion of preovulatory follicles were stimulated to ovulate when hCG was administered early in estrus (25) than when hCG was withheld (7). Administration of hCG after the first preovulatory follicle had ovulated stimulated some preovulatory follicles to ovulate that would otherwise have regressed (8). Therefore, perhaps hCG should have been administered more frequently in the present study. It may also have been beneficial to have used a higher dosage of hCG. Douglas (25) used 4000 IU of hCG, and all the preovulatory follicles ovulated in 8 of 9 mares superovulated using pituitary extract.

Reported embryo recovery rates using conventional techniques vary from 55 to 80% (9,26-28). For both the control and treated cycles, embryo recovery rates were similar to those of reported results but were considerably lower than that achieved by another worker (87% of 15 single ovulating mares) who used a similar 3-minute contact time of the flushing fluid with the endometrium (16). The results in the present study of the percentage of ovulations providing embryos was similar to that reported in 111 cycles (30%) of a previous study (15). Due to the low number of flushes performed in the present study, no conclusions may be drawn from the fact that the embryo recovery rate achieved was lower during treatment with leuprolide acetate than during the control cycle. Nonetheless, it is interesting that only one embryo was recovered from three mares with triple ovulations. A higher embryo recovery rate has been reported from multiple ovulating mares than from single ovulating mares (9). The reason for the poor recovery rate from triple ovulating mares in our study is not clear. However, in women it is known that one problem with superovulation is that the follicles are asynchronous and may yield immature oocytes that cannot be fertilized (22).

In conclusion, in some mares, treatment with leuprolide acetate was successful at inducing a significantly higher number of preovulatory follicles than developed during a control cycle. However, the number of ovulations was not significantly increased and some of the mares remained anovulatory until after the end of treatment. More work is needed to optimize the mode of administration of GnRH agonists and to induce all preovulatory follicles to ovulate.

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physiological solutions (CZB, PBS or isotonic NaCl) with or without 18% (w/v) raffinose before cooling to -20, –50 or –196°C and storage for 1-28 days. On thawing, <2% of spermatozoa frozen with raffinose were partially motile but in other treatments they were immotile and diagnosed as 'dead' by live/dead staining. 95-100% of oocytes injected with sperm heads from spermatozoa frozen with and without raffinose were fertilized normally and 89-100% developed to the 2-cell stage. No differences were found between the physiological media. The majority of oocytes fertilized with spermatozoa frozen in CZB developed to blastocysts (80-94%) but development was significantly reduced after fertilization with spermatozoa frozen in PBS and NaCl especially in the absence of raffinose (69 & 70% *vs* 51& 50%). Normal fertile offspring were obtained in all treatments but there were significantly fewer offspring with spermatozoa stored at -196°C in NaCl with or without raffinose and CZB with raffinose. Testicular spermatozoa were extremely sensitive to cryodamage; ~50% frozen to -196°C in CZB with or without raffinose disintegrated after thawing. Almost 100% of oocytes injected with sperm heads from intact testicular spermatozoa developed to the 2-cell stage but development to blastocysts was reduced significantly compared to controls especially without raffinose. The data indicate that cryopreservation of sperm nuclei require less stringent conditions than for the retention of normal physiological function of intact spermatozoa. Motility and plasma membrane integrity are not essential for fertilization and the production of offspring when nuclei of nonviable spermatozoa are injected into oocytes.

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20. Estimation of lipid and fatty acid composition of zona-intact pig oocytes.

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Lipid composition of unselected pig oocytes (size >75 μm; n = 20,000 to 100,000 per sample) has been reported (Homa *et al*, 1986, J. Reprod. Fertil. 77:425-434), but procedures for smaller samples of selected oocytes are required. The present study used duplicate samples of 1000 zona-intact oocytes (>125 μm) with uniform cytoplasm from oocyte-cumulus complexes (OCC) with good cumulus investment. Prepubertal gilt ovaries, stored in Dulbecco's PBS (Imperial Laboratories, UK) at 37°C, yielded OCC by surface slicing within 2 h post mortem. The OCC were vortexed (10 min) to remove all cumulus cells and selected oocytes were stored (1000 oocytes in 400 μl PBS) in glass vials at -20°C until analysis. After thawing, each sample was homogenised in excess chloroform / methanol (2:1 by vol.) and total lipid extracts were prepared. Thin layer chromatography on silica gel G using hexane / diethyl ether / formic acid solvent (80:20:1 by vol.) separated the 4 lipid classes: phospholipid (PL), triglyceride (TG), cholesterol ester (CE) and free fatty acid (FFA). Following transmethylation of each lipid fraction, gas-liquid chromatography (capillary column, Carbowax; Alltech Ltd., UK) in a Chrompack (Netherlands) instrument determined fatty acid composition, with peaks identified by comparison with standard fatty acid methyl esters. An EZChrom data system (Scientific Software Inc, USA) analysed fatty acid composition and distribution in the 4 lipid classes. Mean ± SEM lipid content was 156 ± 14 μg per sample (~156 ng per oocyte) of which 48 ± 1% was in TG energy stores while PL, the major class of membrane lipids, accounted for 26 ± 1%. CE comprised 11 ± 2% and FFA 16 ± 2%. The most abundant fatty acids in PL, TG and CE were palmitate (C16:0; respective means: 16, 41, 27%), stearate (C18:0; 16, 13, 21%) and oleate (C18:1(n-9); 24, 20, 23%). Polyunsaturates, which contribute to membrane fluidity and can influence development, represented approximately 25% of all fatty acids.

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21. GnRH antagonist treatment delays follicular maturation and the time to the oestradiol peak in the mare.

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Little is known about the hormonal control of follicular growth in the mare. The aim of the present study was to investigate the effects of suppressing gonadotrophins by GnRH antagonist treatment on follicular growth and circulating oestradiol concentrations. The GnRH antagonist Antarelix (100 μg/kg; gifted by Dr R Deghenghi, Europeptides) or vehicle was given subcutaneously to 4 pony mares on day 8 postovulation in successive oestrous cycles. During the treatment and control cycles the mares were blood sampled every 24 hours from the day of ovulation to the following ovulation for measurement of FSH, LH and oestradiol concentrations. The size of the largest follicles was monitored by transrectal ultrasonography. The treatment cycle was longer than the control cycle (32.5 ± 3.8 days *versus* 23.3 ± 1.1 days). GnRH antagonist treatment caused serum concentrations of oestradiol to remain low for an extended period of time after treatment in all 4 mares: number of days from luteolysis (progesterone below 1 ng/ml) to the preovulatory oestradiol surge was 15 ± 3.3 days *versus* 1.8 ± 0.9 days for treatment and control cycles. Similarly the preovulatory increase in FSH and LH concentrations tended to be later in the treatment cycle (FSH: day postovulation 14.5 ± 0.96 and LH: day postovulation 25.5 ± 1.19) than in the control cycle (FSH: day postovulation 10.8 ± 1.25 and LH: day postovulation 19.8 ± 0.75). However, daily FSH and LH concentrations prior to the preovulatory increase surprisingly were not significantly affected by the treatment. Follicular growth followed the pattern of FSH closely, but the follicles did not grow to preovulatory size or produce oestradiol until very late in the treatment cycle *versus* the control cycle. It is possible that the sampling regimen used failed to detect more subtle changes in pulsatility of FSH and LH which could account for the failure of the follicles to grow to preovulatory size until after the effects of the antagonist had waned. This study shows that the stimulus for the follicles to mature and ovulate has been ablated by GnRH antagonist treatment.

ELSEVIER

CONTROL OF FOLLICULAR DEVELOPMENT AND LUTEAL FUNCTION IN THE MARE: EFFECTS OF A GnRH ANTAGONIST

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ABSTRACT

Control of the equine estrous cycle was studied by suppressing gonadotropin secretion by administration of a GnRH antagonist to cyclic pony mares. Four mares received vehicle (control cycle) or a GnRH antagonist, Antarelix™ (100 μg/kg) on Day 8 of diestrus, and blood samples were collected at 15-min intervals from 0 to 16 h, 24 to 36 h, and daily until the next ovulation. Ovarian activity was monitored by transrectal ultrasonography, and measurement of plasma concentrations of progesterone and estradiol. Antagonist treatment eliminated large diestrous pulses of LH. Progesterone concentrations had fallen significantly in all mares by the day after treatment and, in three of the four mares, remained low until luteolysis. However timing of luteolysis (ie., progesterone concentrations <1 ng/mL) was not affected by antagonist treatment. The preovulatory surges of estradiol and LH were significantly delayed in the treatment cycle, as was the appearance of a preovulatory follicle >30 mm. Cycle length was significantly longer during the treatment than the control cycle. These results show that treatment of diestrous mares with a GnRH antagonist attenuated progesterone secretion, indicating a role for LH in control of CL function in the mare, and delayed ovulation presumably because of lack of gonadotropic support.

Key words: mare, GnRH antagonist, follicle, corpus luteum

INTRODUCTION

The luteotropic control of the corpus luteum (CL) varies between species. In women, and old and new world primates, GnRH antagonist treatment has demonstrated that luteinizing

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hormone (LH) drives CL function (9, 13, 18, 39). In ruminants, support of the CL may be more complex, but in general LH is regarded as the primary luteotropin (28). In the mare the control of the CL remains to be elucidated. There is some evidence that LH is luteotropic, because administration of hCG and GnRH to diestrous mares results in increases in plasma progesterone concentrations (22, 23, 38). Also the CL of mares treated with an antiserum against an equine pituitary extract were lighter in weight than those of control mares (32) and appeared, macroscopically, to be regressing (33). However, although it is known that equine luteal cells bind LH (3), no close temporal relationship has been found between pulses of LH and progesterone in peripheral blood of pregnant and nonpregnant mares (31, 38). In vitro studies using short-term incubations of luteal cells have demonstrated either an increase (23) or no change (3, 7) in secretion of progesterone after addition of LH, but it has been shown that results obtained by in vitro culture are affected by the methods used for cell dissociation (3).

GnRH antagonist treatment allows immediate specific suppression of LH and FSH release in vivo and has been used to study pituitary-ovarian relationships in a number of species. No such studies have been described in the mare although the use of a GnRH antagonist has been reported to suppress ovarian activity and synchronize ovulation (30). A close correlation has been demonstrated between hypothalamic GnRH release and pituitary LH and FSH secretion in luteal-phase mares (20), and LH secretion is reduced by 90% to 100% in ovariectomized mares actively immunized against GnRH, showing that LH release in the mare is highly dependent on stimulation by GnRH (15). By contrast FSH is reduced by only 50% in GnRH-immunized mares (15), and therefore it has been suggested that FSH secretion is controlled only in part by GnRH.

In the present study, mares were treated with a potent GnRH antagonist in mid-diestrus at a time when progesterone output by the CL is maximal. This approach to studying the action of LH in vivo in the mare by suppressing its release is complicated by the long half-life of LH in this species (19), which means that plasma concentrations will not drop quickly after antagonist treatment. However, we presumed that the pulsatile release of LH would be eliminated after treatment, which, together with the slow decline in LH, should give an indication of the role of LH in CL support in the mare. Furthermore it was predicted that LH, and possibly FSH, concentrations would be low by the time that follicles should be growing for the mare's next estrus, and so treatment with the antagonist would also give information on the requirement of gonadotropins for folliculogenesis.

MATERIALS AND METHODS

The study was carried out between May and September. In the first instance, three ovariectomized mares (225 to 450 kg) were treated with the GnRH antagonist to determine the effectiveness of the treatment in suppressing gonadotropin release. Blood samples (10 mL) were collected into heparinized evacuated tubes once daily for 7 days before antagonist treatment. On the day of treatment the mare received a subcutaneous injection of the potent water-soluble GnRH antagonist, Antarelix™ ([N-Ac-D-Nal(2)^1, D-pCl-Phe^2, D-Pal(3)^3, D-(HCi)^6, Lys(iPr)^8, D-Ala^{10}] GnRH; 100 μg/kg in 10 mL 5% mannitol, Europeptides, Argenteuil, France). In one mare, daily blood sampling was continued for 7 days, and in the other two mares samples were collected for 32 days after antagonist treatment.

To study the effect of the antagonist on intact mares, four cyclic pony mares aged 7 to 18 years and weighing 198 to 298 kg were used. The ovaries of the mares were examined daily by transrectal ultrasonography during estrus until the day of ovulation (Day 0). The experiment was carried out during two cycles. In the first cycle, on Day 8 after ovulation, the mares received a subcutaneous injection of vehicle (10 mL 5% mannitol). In the second cycle the same mares were given a subcutaneous injection of the GnRH antagonist (100 μg/kg) on Day 8 after ovulation. Blood samples (20 mL) were collected via indwelling jugular cannulae into evacuated heparinized tubes at 15-min intervals for 16 h (Day 8) and also between 24 and 36 h (Day 9) after injection of either the drug or the vehicle. Blood samples were then collected daily by venipuncture until they returned to estrus and ovulated. The diameter of the CL and follicular activity were monitored by transrectal ultrasonography on every second day throughout the cycle until estrus, when the mares were scanned daily.

Blood samples were kept on ice until being centrifuged at 2000 x g for 15 min at 4°C. Plasma was decanted and stored at -20°C until assay. Daily samples and samples collected every hour during the sampling periods on Days 8 and 9 were assayed for progesterone. Daily samples were assayed for estradiol-17β and FSH, and LH was measured in all samples including the samples collected at 15-min intervals.

Radioimmunoassays

With the exception of estradiol, concentrations of hormones were measured by radioimmunoassay directly in plasma without extraction using techniques previously validated for LH (38), progesterone (8, 24) and FSH (34). For estradiol-17β, samples were extracted once in diethyl ether and assayed as previously described (17). The mean extraction efficiency for estradiol was 88% ± 1.1% (SEM.). In the FSH assay, a different rabbit anti-human FSH antibody (NIDDK-anti-hFSH-6) to that reported previously was used, at a final dilution of 1:10,000. The main cross-reactivity of this antibody was the hTSH (1%). The main cross-reactivities of the progesterone antiserum were with 5-pregnan-3,20 dione (9.5%), 11-deoxycorticosterone (6.2%) and 17-hydroxyprogesterone (3.4%). Progesterone standards were prepared in ovariectomized mare plasma. Assay sensitivities were 0.6 ng/mL for LH, 0.5 ng/mL for progesterone, 8 pg/mL for estradiol and 0.5 ng/mL for FSH, with intra- and interassay coefficients of variation of 13.8% and 14% for LH, 9.0% and 12.6% for progesterone, 4.6% and 7.8% for estradiol, and 6.3% and 10.2% for FSH, respectively. For each hormone, displacement curves produced by serial dilutions of plasma and spiking of samples containing low concentrations of the respective hormone were parallel to the standard curves. The average recovery of a known amount of hormone added to equine plasma was 92% for LH, 100% for progesterone, 91% for estradiol and 94% for FSH.

Statistical Analyses

Differences in cycle length, CL diameter until luteolysis, progesterone concentrations in hourly samples collected on Days 8 and 9, and mean LH concentrations in the serial blood collections were compared using the Kruskal Wallis Analysis of Variance. Luteolysis was defined as starting when progesterone declined to concentrations below 1 ng/mL. Daily progesterone concentrations in control and treatment cycles were compared using the Wilcoxon

signed rank test. Pulses of LH were identified as previously described (38). Differences in time to a preovulatory rise in gonadotropin and estradiol concentrations were compared by the Kruskal Wallis Analysis of Variance. The start of the preovulatory rise was defined as the day when concentrations first exceeded mean +1 SEM of daily concentrations for each mare, and when the increase was maintained for at least 3 days.

RESULTS

In the ovariectomized pony mares, mean concentrations of FSH and LH had fallen to approximately 40% and 10% of pretreatment levels, respectively, by Day 3 or 4 after antagonist treatment and remained at this level for around 7 days. By 32 days after antagonist treatment, concentrations of LH were still only around 50% of those collected before treatment, but FSH had returned to pretreatment concentrations.

Estrous cycles of the intact mares were longer during the treatment than the control cycle (33.5 $\pm$ 3.8 days vs. 24.0 $\pm$ 1.1 days; P = 0.01). The major factor in lengthening of the treatment cycle was caused by extension of the follicular phase (time between luteolysis and ovulation 17.5 $\pm$ 3.1 days vs. 6.0 $\pm$ 0.9 days; P<0.01) rather than by premature luteolysis. The prolongation of time to ovulation was due to a delay (P<0.05) in appearance of a large preovulatory follicle (>30 mm) on the ovaries during the treatment compared with the control cycle (Day 27.5 $\pm$ 4.2 vs. 18.8 $\pm$ 0.9; Figure 1), rather than the actual time taken for this follicle to grow and ovulate as determined by ultrasonography (5.8 $\pm$ 1.1 days vs. 5.5 $\pm$ 0.7 days). The diameter of the CL tended to be less between treatment (Day 8) and luteolysis (progesterone <1 ng/mL) in the treatment cycle (21.3 $\pm$ 2.6 mm) than during the control cycle (27.0 $\pm$ 2.5 mm), but this failed to reach significance (P = 0.16).

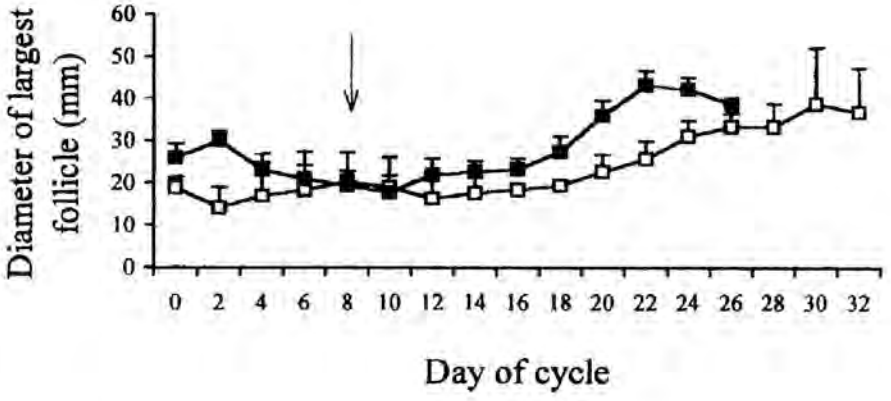

Figure 1. Mean ($\pm$ SEM) diameter of the largest ovarian follicle in 4 mares during a control (solid squares) and treatment (open squares) cycle. Arrow shows day of GnRH antagonist treatment.

In three of the mares, daily progesterone concentrations were significantly lower ($P<0.01$) after antagonist treatment compared to the control cycle (Figure 2). The decrease in progesterone concentrations was significant in the mean of the hourly samples of all 4 mares collected on the day after antagonist treatment (11.4 ± 2.5 ng/mL vs. 7.6 ± 2.2 ng/mL; $P = 0.01$) compared with the control cycle (11.2 ± 1.6 ng/mL vs. 10.6 ± 1.1 ng/mL). In the mare in which a decrease in progesterone concentrations was not maintained after antagonist treatment, a 40-mm follicle that had fibrin tags in the antrum and a thick echogenic wall was recorded from Day 5 of the treatment cycle. This follicle persisted throughout the cycle, regressing by Day 16. The progesterone concentrations in this mare were considerably higher than those in the other mares, and, although there was a decline in progesterone concentrations on Day 9 comparable to other mares, the levels rose again by Day 10 and remained high (16 to 23 ng/mL) until Day 15. Concentrations fell to below 1 ng/mL on Day 18. Results from this mare were excluded from the analysis of mean daily progesterone samples after treatment.

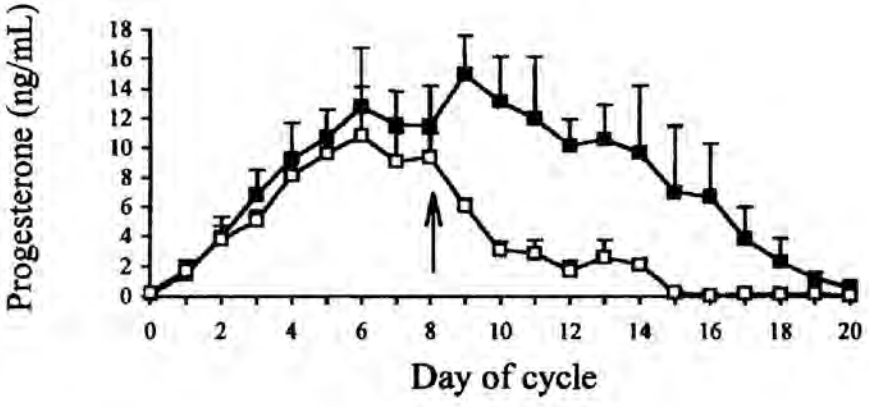

Figure 2. Mean (± SEM) plasma progesterone concentrations in 3 mares (excludes a mare with an anovulatory luteinized follicle) for 20 days after ovulation in the control (solid square) and GnRH antagonist treatment (open square) cycle. Arrow shows day of treatment.

Concentrations of FSH in daily samples after antagonist treatment were not significantly different in treatment and control cycles, but the start of the diestrous peak tended to be later ($P=0.07$) in treatment (Day 14.5 ± 1.0) than in control (Day 10.8 ± 1.3) cycles (Figure 3). Daily LH concentrations were very variable among mares and cycles. Mean concentrations after antagonist treatment were not significantly lower than during the control cycle.

Concentrations of LH started rising for the preovulatory surge at a significantly ($P<0.01$) later time during the treatment cycle (Day 25.5 ± 1.2) than during the control cycle (Day 19.8 ± 0.8; Figure 4). In the serial samples, mean LH was similar in both groups on Day 8 (2.4 ± 0.3 ng/mL and 2.2 ± 0.3 ng/mL in the control and treatment cycles, respectively), but tended to be

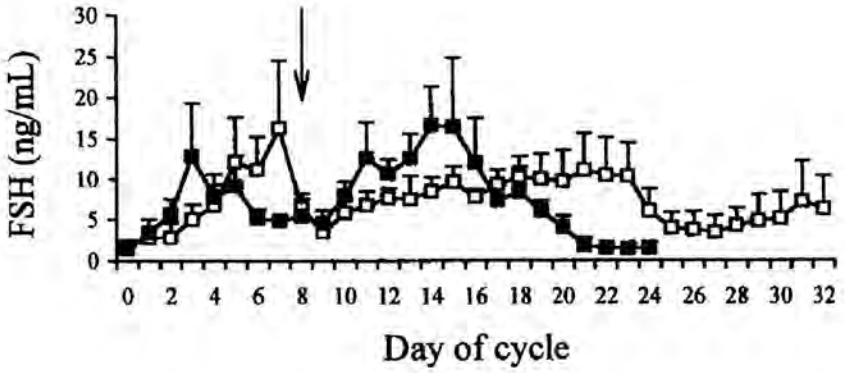

Figure 3. Mean (± SEM) concentrations of FSH in 4 mares during a control (solid squares) and GnRH antagonist treatment (open squares) cycle. Arrow shows day of treatment.

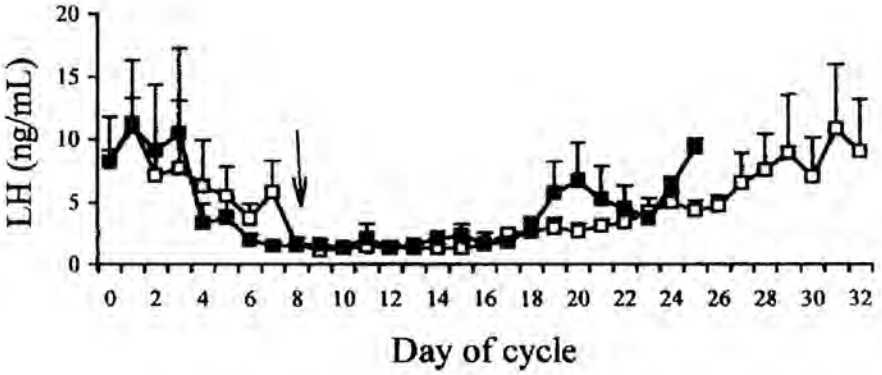

Figure 4. Mean (± SEM) concentrations of LH in 4 mares during a control (solid squares) and GnRH antagonist treatment (open squares) cycle. Arrow shows day of treatment.

lower (P=0.12) on Day 9 in the treatment cycle (1.3 ± 0.4 ng/mL) than in the control cycle (1.9 ± 0.5 ng/mL). On Day 8, 2 mares, and on Day 9, one mare had one high-amplitude pulse of LH during the control cycle, whereas none of the mares had high-amplitude LH pulses on either day during the treatment cycle.

In 1 of the 8 cycles (treated + control) studied there were two peaks of estradiol—the first in early diestrus and the second at estrus. In the remaining cycles the estradiol surge occurred at estrus. The surge was significantly (P=0.01) delayed in the treatment cycle (Day 28.8 + 4.6) compared with the control (Day 19.0 ± 1.4) cycle (Figure 5). Mean daily concentrations after antagonist treatment tended (P=0.08) to be lower in the treatment cycle (3.6 ± 0.4 pg/mL) than in the control cycle (7.5 ± 1.4 pg/mL).

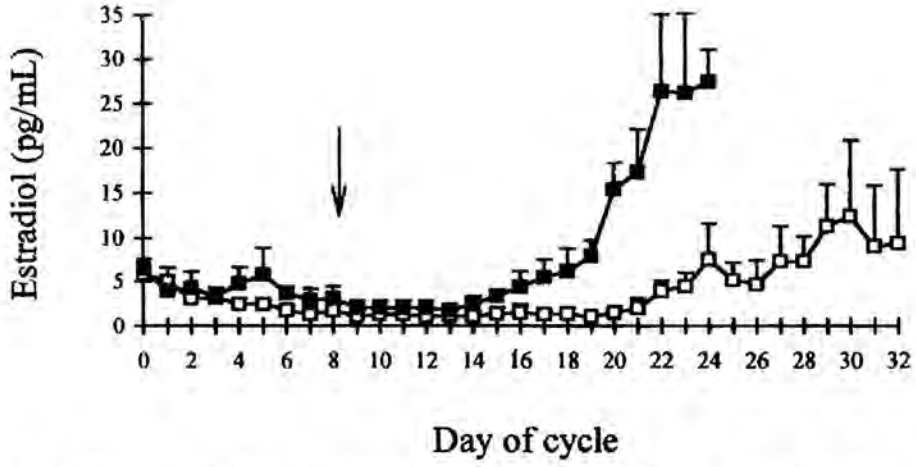

Figure 5. Mean (± SEM) concentrations of estradiol in 4 mares during a control (solid squares) and GnRH antagonist treatment (open squares) cycle. Arrow shows day of treatment.

DISCUSSION

Treatment of diestrous mares with a potent GnRH antagonist resulted in attenuation of progesterone secretion, indicating a role for LH in support of the mare CL. Although this occurred in the apparent absence of a decline in plasma concentrations of LH, it should be noted that in these mares, diestrous basal LH concentrations were very low and changes may have been too subtle to detect with our radioimmunoassay. Confirmation that the treatment reduced pituitary gonadotropin output was obtained from the ovariectomized mares, in which the initial LH and FSH concentrations were considerably higher than in the intact mares Treatment with GnRH antagonists in other species usually results in profound suppression of LH secretion within 4 hours of administration (5, 18, 39). However the long half-life of LH in the mare (approximately 5 hours; 19) compared with less than 1 hour in other species meant there was a delay in reaching very low serum concentrations after GnRH-antagonist treatment. Pituitary release was suppressed for a prolonged period of time in ovariectomized mares after a single subcutaneous injection of antagonist, suppression of LH being greater than that of FSH.

High-amplitude pulses of LH were not measured in any of the cyclic mares after antagonist treatment. Between Days 4 and 12 of diestrus, the mean frequency of LH pulses was found to be only 1.6 per day (21), which may explain why our sampling regimen failed to detect pulses in one of the control cycles. However the immediate drop in progesterone after GnRH-antagonist treatment in the present study indicates the importance of pulsatility of LH secretion in the maintenance of CL function in the mare. Interestingly, episodic secretion of progesterone was maintained on Days 8 and 9 in mares treated with antagonist (data not shown) despite the absence of high-amplitude LH pulses. This confirms the lack of correlation found between LH and progesterone pulses in mares (31, 38) and suggests the presence of an autonomous pulse-generating system within luteal tissue as described in the cow (35) and ewe (25). It is thought that FSH does not act as a luteotropin because of the low level of binding of FSH to the equine CL (37), and therefore it is unlikely that the effect seen on progesterone concentrations was caused by reduction in FSH concentrations after treatment.

The established mode of action of the GnRH antagonist is to block the GnRH receptors of the pituitary selectively (5). It is unlikely that the antagonist blocked a direct luteotropic action of GnRH on the CL. Receptors for GnRH have been found in the rat CL (6), but results from other species indicate that GnRH probably does not have a direct effect at the ovarian level (4, 14).

Diameter of the CL after antagonist treatment tended to be less than in the control cycle. Similarly, in a previous study, mares treated with antiserum against LH had CL that were lighter in weight than those of control mares (33). After GnRH-antagonist treatment, progesterone concentrations had fallen to relatively low levels by 48 h after treatment. After PGF-2α treatment, progesterone decreases to less than 1 ng/mL within 48 h (29). Therefore the timescale was similar to PGF-2α-induced luteolysis, although with the GnRH antagonist, luteolysis was incomplete. Complete luteolysis did not occur significantly earlier in the treatment cycle than in the control cycle. By contrast, administration of Antarelix™ to marmoset monkeys on Day 9 of the luteal phase resulted in luteolysis within 24 hours (40). The failure of the antagonist to cause luteolysis in mares indicates that progesterone secretion was supported either by basal LH concentrations or by some other luteotropin.

It has been reported that two broad surges of FSH occur at 10- to 12-day intervals during the estrous cycle of the mare (10). One of the surges occurs during late estrus and early diestrus and the other in late diestrus. However there is little consistency in patterns among individual animals (16), and no late estrous surge was seen in any of our mares. A recent paper by Irvine and coworkers (21) shows that sampling one time per day for FSH concentrations may be misleading because of the markedly pulsatile secretion. In general FSH concentrations in our mares were low throughout estrus and elevated in diestrus. The late diestrous surge of FSH in the mare appears to be associated with selection and growth of a dominant follicle (1, 2, 11, 26). In other species the suppressive effect of GnRH-antagonist treatment on FSH secretion is less than for LH (5). However antagonist treatment appeared to delay the diestrous rise in FSH in the intact mares as previously reported (30). Suppression of FSH and/or LH by administration of either charcoal-stripped follicular fluid or antiserum against a crude gonadotropin preparation, active immunization against LHRH (36) or chronic GnRH agonist treatment inhibits follicular development in the mare (1, 32). In the present study the delay in appearance of the preovulatory increase in LH and the diestrous increase in FSH in the treatment cycle may

account for the retarded follicular growth. However, precise information on gonadotropic control of folliculogenesis in the mare is lacking, and we did not study pulsatility of gonadotropin release in the preovulatory period. Equine follicles can develop to preovulatory size under a variety of FSH patterns (16). However, it seems that maturation of the follicle requires LH or FSH plus LH, and it is thought that LH is required for the prolonged maturation of the previously primed preovulatory follicle during estrus, when FSH concentrations are low (10). The results from our study where follicular development was significantly delayed by GnRH-antagonist treatment indicate a role for gonadotropins in preovulatory follicular growth and maturation.

The mare that failed to show a sustained drop in progesterone after antagonist treatment had a large 40-mm anovulatory follicle from Day 5 after ovulation that showed ultrasonographic evidence of luteinization and persisted until the end of diestrus. This mare also had very high concentrations of progesterone during this cycle compared with the other mares, suggesting that the anovulatory follicle was indeed producing progesterone. Failure of this mare to respond to the GnRH antagonist indicates that this luteinized follicle was not under the same luteotropic control as a CL. The etiology and incidence of formation of luteinized anovulatory follicles in nonpregnant mares is not known, but it would appear that either there is insufficient gonadotropic stimulation to induce ovulation or the follicle fails to respond to gonadotropin stimulation.

In conclusion the results indicate that high-amplitude LH pulses are involved in maintaining progesterone production by the equine CL. The delay in follicle growth and ovulation in mares treated with a GnRH antagonist confirms a role for gonadotropins in preovulatory follicle development.

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GONADOTROPIC CONTROL OF FOLLICULAR GROWTH IN THE MARE

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INTRODUCTION

Tonic FSH and LH stimulation can support follicles to an immature antral stage but larger follicles are dependent on pulsatile gonadotropin (GTN) supply [1] [2]. Additional stimulation by FSH, and later LH, is required for further growth. FSH alone will support bovine follicles until they reach a diameter of 9 mm, when LH becomes necessary for the continued development and growth of the follicle [2]. If GNT support is withdrawn by hypophysectomy, antral follicles will become atretic in sheep [3]. Down-regulation of LH with a GnRH agonist in mares has been reported previously [4] [5]. In both studies GnRH agonist administration resulted in suppressed LH concentrations and an increased interovulatory interval. Neither of these studies reported the effect on FSH or the growth patterns of smaller follicles. The aims of the present study were to investigate 1. whether constant administration of a GnRH agonist would suppress the secretion of FSH as well as LH and 2. the effect of down-regulating GTNs on follicular development.

MATERIALS AND METHODS

Six mares, weighing 383 – 543 kg and aged 8 – 16 years, were used. Follicular activity was monitored by daily ultrasound scanning from 10 days before the first ovulation until ovulation at the end of the trial. On day 6 after the first ovulation, the mares received GnRH agonist implants sc at a dose rate of 4.0 μg goserelin/kg/day (Zoladex ICI 118630, goserelin acetate; 3.6 mg implants, Zeneca Pharmaceuticals, Macclesfield, Cheshire, UK). After 28 days (day 34) the mares received new implants, again at a dose rate of 4.0 μg/kg/day for 28 days. Blood samples were collected once daily. Plasma was assayed for FSH and LH [6]. For each mare the dominant follicle at the beginning and the end of the cycle was tracked retrospectively. The subordinate follicles were ranked from largest to smallest without regard to day to day identity of the individual follicles. The ranked follicles were divided into groups with the 4 largest follicles per mare in group 1, the 4 next largest follicles in group 2, until all follicles had been designated. When plotted against days, mean diameter of a particular group on a particular day was used.

RESULTS

The mean interovulatory interval was 98 ± 6 days (range 79 – 125). Three mares either ovulated or had follicles that luteinized 10 – 12 days after insertion of the GnRH agonist implant. Concentrations of LH increased on the day after the insertion of the first set of implants, but following this concentrations remained below 5 ng/ml for a prolonged period of time (Fig 1). FSH

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dropped shortly after implant insertion and stayed below 4 ng/ml until day 78 (Fig 1). Follicle sizes reached a minimum after the second administration of implants, with no follicles larger than 10 mm between days 37 and 64 (Fig 2).

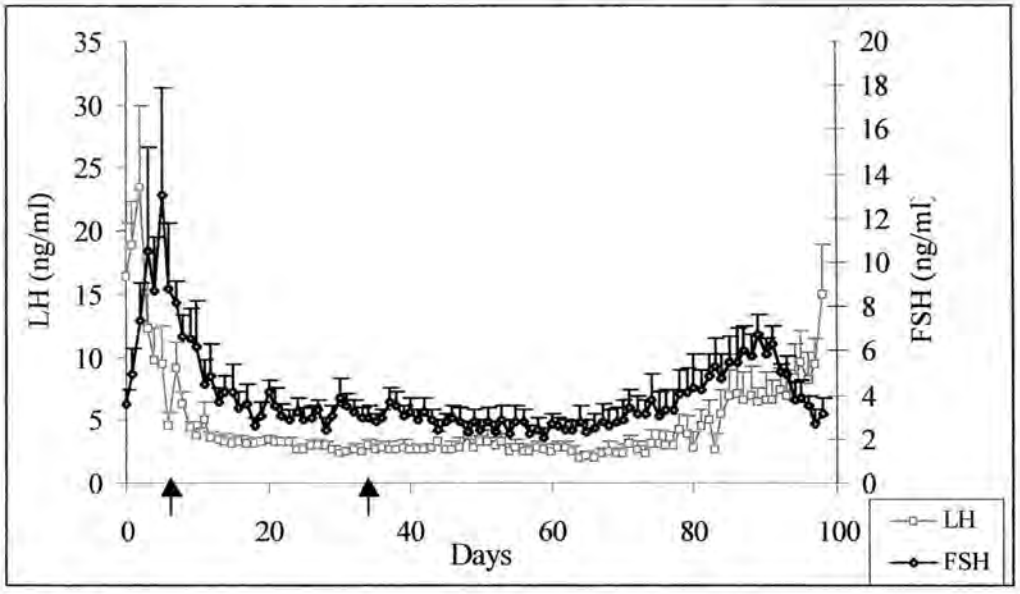

Fig 1. Effect of a GnRH agonist (Zoladex) on LH and FSH concentrations (mean + SEM, n = 6 mares). Black arrows denote insertion of GnRH agonist implants on days 6 and 34.

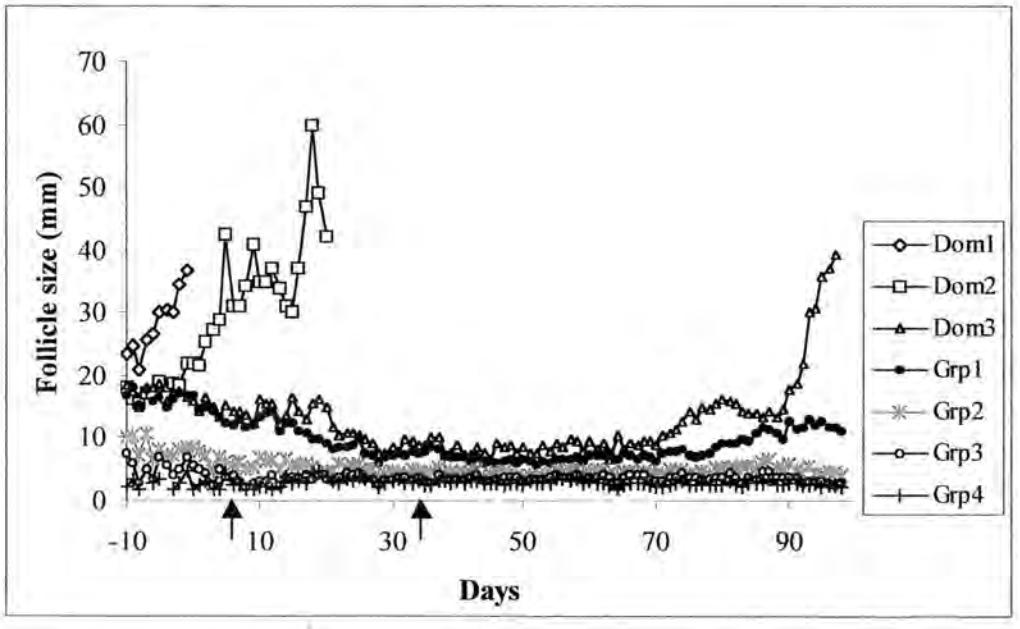

Figure 2. Effect of a GnRH agonist (Zoladex) on follicle growth (n = 6 mares). Black arrows denote day of insertion of GnRH agonist implants. Dom 1: Follicles which ovulated on day 0. Dom 2: Follicles which either ovulated or luteinized after insertion of implants. Dom 3: follicles which ovulated at the end of the trial period. Grp 1: group of 4 largest subordinate follicles from each mare. Grp 2 – 4: groups of progressively smaller follicles with each mare contributing with 4 follicles each per day.

DISCUSSION

The present study demonstrated that continuous administration of goserelin could suppress gonadotropin release and that equine follicles up to 10 mm are relatively independent of GTNs. Early follicular development can progress without continual GTN stimulation, but a reduction in GTN concentrations at later stages of development impairs continued folliculogenesis. The lack of FSH prevented follicle growth beyond 10 mm, resulting in a drastically prolonged mean interovulatory interval. Once the ovulatory follicles did start growing after the implants stopped releasing GnRH, the growth rate was faster than the growth rate of the preovulatory follicle before the trial started. The size of the ovulatory follicle was similar to that of the ovulatory follicle before the trial started. The changes in FSH concentration were closely mirrored by changes in follicle sizes demonstrating the dependency of small follicles upon FSH in the present study. If FSH support is withdrawn by hypophysectomy, all antral follicles will become atretic in sheep [3]. Whether complete removal of FSH and LH in mares will cause follicles to stop growing at an even smaller size remains to be seen. Studies in sheep showed that there was no follicle size difference in down-regulated sheep compared to hypophysectomised sheep [1] [3]. In the present study there was an early increase in LH as a response to the GnRH agonist, but an increase in FSH was not detected with daily sampling. In a pilot study, intense sampling around the time of implant insertion showed a marked increase in both LH and FSH, which lasted approximately 24 hours. FSH and LH gradually increased after day 62, which corresponded with the predicted time that the implants would stop releasing goserelin. No follicle reached 14 mm until day 75, indicating a delay from the rise in GTNs until follicle growth beyond 10 mm was apparent. Whether the new dominant follicles were from the pool of antral follicles already present on the ovary or whether they were recruited from the preantral follicles is not known. In conclusion, equine follicles are dependent upon FSH and LH for growth beyond 10 mm. The present experimental model may be a useful tool in further investigation of regulation of follicle growth and atresia.

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Apoptosis in equine granulosa cells and its relationship to cumulus expansion and oocyte chromatin configuration in ovarian follicles

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During the oestrous cycle follicles grow and either ovulate or regress. Regressing follicles undergo atresia and in many species apoptosis has been identified as the underlying mechanism in this process. The aims of this study were to establish whether equine granulosa cells degenerate via an apoptotic mechanism and whether the presence of apoptotic cell death in granulosa cells is correlated with oocyte quality. Ovaries from mares at unknown stages of the oestrous cycle were obtained from an abattoir. In Expt 1, follicles (n = 352) from 37 mares were processed. DNA was extracted from granulosa cells, fractionated by agarose gel electrophoresis, stained with ethidium bromide and visualized with ultraviolet light or end-labelled with [^{32}P]dideoxy-ATP, and autoradiography was performed after electrophoresis. In Expt 2, follicles (n = 34) from four mares with at least one follicle > 35 mm in diameter were processed. DNA was extracted from the granulosa cells; the cumulus oophorus was classified and the oocyte chromatin was stained with Hoechst 33258 fluorescent stain. In Expt 1, apoptosis, as determined by the characteristic laddering of internucleosomal DNA fragments, was present in 45% of all follicles. Apoptosis was apparent primarily in follicles < 20 mm in diameter and was present with greatest frequency in follicles 6–10 mm in diameter. Apoptosis was not detected in follicles > 27 mm in diameter. The presence of sheared DNA of a wide range of different molecular masses, possibly indicative of necrotic cell death, was positively correlated with follicle size. In Expt 2, apoptosis was detected in 50% of follicles < 20 mm in diameter but not in follicles > 30 mm in diameter. The oocytes had an expanded cumulus oophorus in 58% of follicles < 20 mm in diameter, whereas 80% of follicles > 30 mm in diameter had a compact cumulus oophorus. In these mares, follicles < 20 mm in diameter appeared to undergo apoptotic changes as well as cumulus expansion. These findings indicate that degeneration occurs in many follicles that are not destined for ovulation and that detection of apoptosis can be used as an indicator of follicular degeneration in mares. Apoptosis, as a marker of cell death, can be used to study the growth and selection of follicles, and to correlate the health of granulosa cells with oocyte quality.

Introduction

During the oestrous cycle, most growing follicles will regress and only a few will proceed to ovulation. Driancourt *et al.* (1982a) reported that there are approximately 35 000 primordial follicles in the mare ovary, of which 100 are growing at any given time. Only 1–2 follicles ovulate each cycle and thus most of the follicles in the ovary undergo atresia. Atresia is defined as the presence of pyknotic nuclei in histological sections of follicles (Kenney *et al.*, 1979). The onset of atresia in a follicle is difficult to determine as it is characterized in only a small section of the follicular wall and because pyknosis is seen to some extent even in dominant viable follicles (Ireland and Roche, 1982).

In most mammals, the majority of follicles undergo atresia during the late preantral to early antral stages when continued growth is dependent upon gonadotrophins (Tilly, 1996). In mares, atresia is observed primarily in follicles > 1.5 mm in diameter (small antral) but not in preantral follicles (< 0.3 mm in diameter) (Driancourt *et al.*, 1982b).

The underlying mechanism in follicular atresia is apoptotic cell death. This has been observed in rats (Nahum *et al.*, 1996; Boone and Tsang, 1997), sheep (Jolly *et al.*, 1997), cattle (Jolly *et al.*, 1994) and pigs (Guthrie *et al.*, 1994). Apoptosis is a form of programmed physiological cell death. This is in contrast to necrosis which is 'accidental' (pathological) cell death caused by a wide variety of harmful conditions (Kerr *et al.*, 1972; Schwartzman and Cidlowski, 1993; Darzynkiewicz *et al.*, 1998). Morphologically, apoptosis is characterized by loss of cell volume (cytoplasmic condensation) and nuclear pyknosis resulting from margination of the chromatin and its redistribution against the nuclear envelope (Kerr *et al.*, 1972). Biochemically, apoptosis is characterized by loss of DNA integrity after endonuclease-mediated fragmentation (Wyllie, 1980). The endonuclease responsible for apoptotic DNA degradation is a caspase-activated deoxyribonuclease (CAD) (Enari *et al.*, 1998). The internucleosomal cleavage of DNA results in high molecular mass fragments (oligonucleosomes, 180–200 bp), which appear as 'DNA ladders' when visualized by agarose gel electrophoresis (Wyllie, 1980; Tilly *et al.*, 1991). In contrast, necrosis is characterized by random endonuclease degradation of DNA which produces a continuous spectrum of DNA fragments of different sizes that appear as a smear after electrophoresis on an agarose gel (Afanasev *et al.*, 1986; Gold *et al.*, 1994).

It is not known whether DNA laddering in agarose gels can be recognized before histological changes are visible in the follicles of mares, but in rats DNA fragmentation can be detected 24 h before morphological indicators of atresia are visible (Nahum *et al.*, 1996). Histologically, it may not be possible to detect the early stages of apoptosis, as this technique involves evaluation of nuclear chromatin condensation that occurs in the final stages of apoptosis. In cows and ewes, apoptotic death of granulosa cells and mitosis may occur within the same follicle, so atresia of a follicle is determined by a dynamic equilibrium between cell division, differentiation and death (Jolly *et al.*, 1994, 1997).

Growing follicles maintain oocytes in meiotic arrest. The oocytes are affected only in the last stages of atresia. Oocytes within atretic follicles may undergo maturation or meiosis like alterations and the cumulus oophorus may expand. Oocytes with an expanded cumulus are more likely to be recovered from atretic follicles and it is thought that dying follicles lose the ability to suppress oocyte maturation (Hinrichs and Williams, 1997). The aims of the present study were to establish whether equine granulosa cells degenerate via an apoptotic mechanism and whether apoptotic cell death in granulosa cells is correlated with specific oocyte characteristics (chromosome configuration and cumulus oophorus expansion) in mares.

Materials and Methods

The study was performed during the breeding season of 1997 (May–October). Ovaries of cyclic mares at unknown stages of the oestrous cycle were obtained from an abattoir.

Collection of ovaries and follicle processing

The ovaries were kept at 30–35°C in a solution of medium 199 (M199) with Hank's salts and 25 mmol Hepes l^{-1} (Gibco BRL, Life Technologies Ltd, Paisley) for 1–6 h. All follicles > 10 mm in diameter were processed. Smaller follicles that were discovered on the ovarian surface or after cutting into the stroma were also processed. Before aspiration each follicle was measured with a ruler. Each follicle was cut open and the inner wall was scraped with a bone curette. The granulosa cells were flushed off using a solution of M199 with Hank's salts. The granulosa cells were pelleted by centrifugation at 2000 *g* for 5 min at 4°C, snap frozen and stored at –70°C until DNA extraction was performed.

DNA extraction

Granulosa cells were lysed with 0.5% SDS, 0.1 mol NaCl l^{-1}, 0.05 mol Tris l^{-1} (pH 8.0) and 2.4 mmol EDTA l^{-1} and digested with 100 μg proteinase K ml^{-1} at 57°C for 6 h. Protein precipitation was initiated with 8 mol potassium acetate l^{-1} (75 μl) and chloroform (0.5 ml), either at 5°C for 1 h or at –20°C overnight. After centrifugation for 8 min at 10 000 *g* at 4°C, further extraction and precipitation were performed with isopropanol (0.5 ml) for 2 h at –70°C, followed by precipitation in 80% (v/v) ethanol (1 ml). After removal of the ethanol, the samples were air-dried, resuspended in deionized filter-sterilized H_2O and stored at –20°C. The DNA concentration of each sample was quantified spectrophotometrically by reading the absorbance at 260 and 280 nm.

DNA analysis

In samples that contained ≥ 10 μg DNA, the DNA fractions were separated by agarose gel electrophoresis, using 10 μg DNA from a single follicle in each lane of a 1.5% (w/v) agarose gel. Ethidium bromide (1 mg ml^{-1}) was incorporated into the agarose gel to stain the DNA and the DNA was visualized using an ultraviolet transilluminator. DNA from freshly isolated murine thymocytes was used as a negative (non-apoptotic) control and DNA from murine thymocytes incubated for 24 h with 100 μg dexamethasone ml^{-1} (Sigma-Aldrich Company Ltd, Poole) was used as a positive (apoptotic) control (Cohen and Duke, 1984).

Samples containing 2–10 μg DNA were analysed by 3′-end labelling. The DNA samples were labelled with 2 μl [^{32}P]dideoxy-ATP (50 μCi) (Amersham Life Science Ltd, Little Chalfont) at the 3′-end by incubation for 60 min at 37°C with 1 μl terminal transferase (Sigma-Aldrich Company Ltd), 10 μl reaction buffer (1 mol NaCl l^{-1}, 0.125 mol Tris HCl l^{-1} and 0.125% (w/v) BSA (Sigma-Aldrich Company Ltd)) and 5 μl 10 mmol cobalt chloride l^{-1} (Sigma-Aldrich Company Ltd). The incubation was terminated using EDTA and the samples were centrifuged on spin columns (Boehringer Mannheim, Lewes) for 3 min at 1000 *g* to purify the radiolabelled DNA. The labelled DNA was separated by electrophoresis, vacuum dried and exposed to Kodak X-ray film.

Follicles were classified as apoptotic when the characteristic laddering of internucleosomal DNA fragments was detected (Wyllie, 1980). Follicles were classified as containing granulosa cells degraded in a way that may be indicative of necrosis when the DNA did not show the internucleosomal cleavage pattern of apoptosis, but showed a distinct smearing of DNA fragments of a range of different molecular masses down the gel lane. Follicles were classified as viable when the DNA did not smear or ladder when separated on an agarose gel (Fig. 1).

Cumulus oophorus and oocyte chromatin evaluation

Four mares that had at least one follicle > 35 mm in diameter were selected to obtain a wide range of follicle sizes. The cumulus oophorus, oocytes and apoptosis in granulosa cells were evaluated. The internal follicle wall was scraped to release granulosa cells and the cumulus oophorus with the oocyte. Each cumulus oophorus was examined using a stereomicroscope and the oocytes were denuded by pipetting with pipettes that had successively smaller diameters (0.2–0.6 mm) in a solution of 0.25% (w/v) trypsin (Gibco). The denuded oocytes were fixed with 4% (v/v) buffered formol saline and then mounted on a slide with 8 ml glycerol and PBS (3:1) with 2.5 μg Hoechst stain 33258 ml^{-1} (Sigma-Aldrich Company Ltd). The chromatin configuration was evaluated under a fluorescence microscope at × 400.

Cumulus oophorus morphology was classified as either compact, expanded or denuded using the criteria of Hinrichs *et al.* (1993a). Briefly, a compact cumulus oophorus has tight granulosa cells over and around the oocyte and is smaller than an expanded cumulus, in which the granulosa cells are connected more loosely, there is more matrix between the granulosa cells and there may be a yellow hue around the oocyte. Denuded oocytes lack part or all of the cumuli oophori.

The chromatin configuration was classified as follows using the criteria of Hinrichs *et al.* (1993b): fluorescent nucleus, condensed chromatin, metaphase I and II, and abnormal configurations.

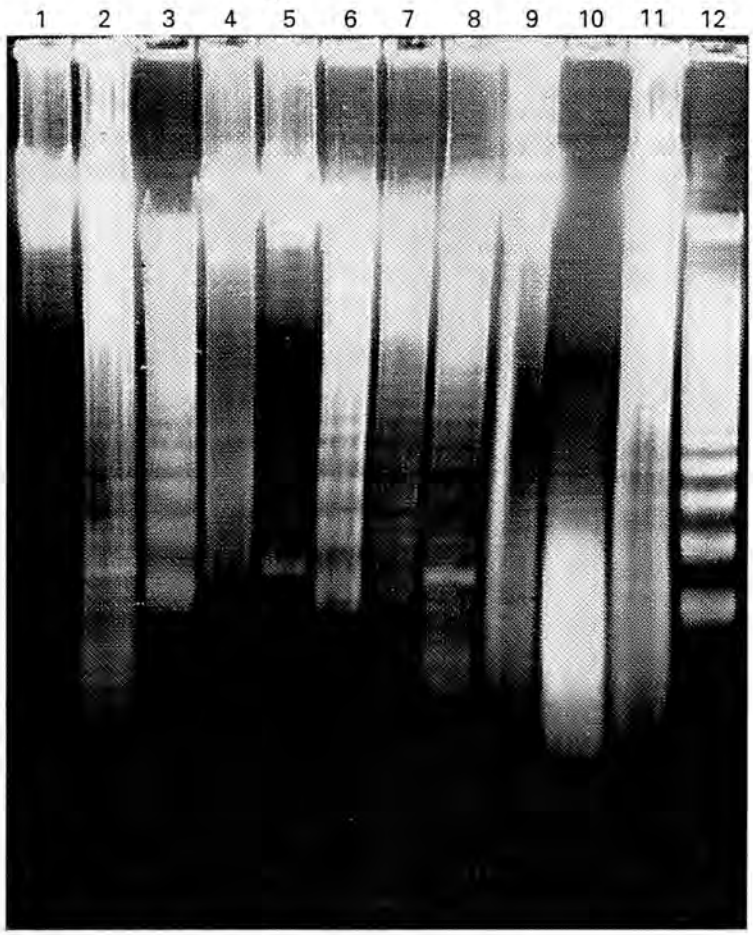

Fig. 1. DNA extracted from granulosa cells from representative equine follicles resolved by agarose gel electrophoresis. Lane 1: negative control (DNA from freshly isolated murine thymocytes); lane 12: positive control (DNA from murine thymocytes incubated for 24 h with 100 μg dexamethasome ml^{-1}). Apoptosis, seen as laddering of the DNA, is observed in lanes 2, 3, 6–8 and 11. Low molecular mass smearing is present in lanes 4, 9 and 10 (this may indicate necrosis). Lane 5 contains DNA from a healthy equine follicle.

Oocytes with a fluorescent nucleus contain a diffusely stained germinal vesicle. In oocytes with condensed chromatin, the chromatin is contracted into a dense mass. Oocytes are classified as having abnormal configurations when clumps of chromatin are scattered randomly within the oocytes.

Results

In Expt 1, 352 follicles from 37 mares, ranging from 2 to 60 mm in diameter were divided into seven size groups (Table 1). Ninety-three per cent of follicles contained enough DNA for either agarose gel electrophoresis or DNA 3'-end-labelling. The other follicles were either too small to obtain sufficient granulosa cells, or the granulosa cell layer was very thin and few granulosa cells were present. In 21% of follicles, no apoptosis or smearing of the DNA was observed and these were classified as

Table 1. Distribution of equine follicles in size groups in Expt 1

Follicle diameter (mm)	Number of follicles (%)
2–5	68 (19)
6–10	122 (35)
11–15	81 (23)
16–20	38 (11)
21–25	22 (6)
26–30	8 (2)
31–60	13 (4)

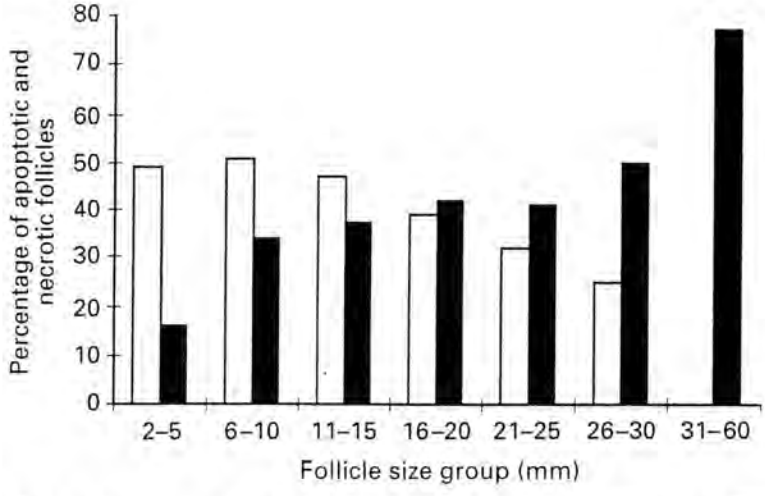

Fig. 2. Percentage distribution of apoptosis (❑) and smearing (■), which is indicative of necrosis, in equine follicles of different sizes.

viable. In 34% of follicles, the granulosa cells did not show the internucleosomal cleavage pattern, but the DNA was cut into a range of different sizes. Forty-five per cent of follicles were apoptotic. Apoptosis was evident primarily in follicles < 20 mm in diameter, of which most were 6–10 mm in diameter. Apoptosis was not detected in follicles > 27 mm in diameter. Severe smearing was observed for 38% of follicles > 31 mm in diameter and in only 7% of smaller follicles (Fig. 2).

In Expt 2, 34 follicles from four mares with at least one follicle > 35 mm in diameter, ranging from 3 to 60 mm in diameter, were divided into two groups: group A contained 26 follicles (3–20 mm in diameter) and group B contained seven follicles (31–60 mm in diameter). There was only one follicle between 21 and 30 mm in diameter and it was excluded from further analysis. Oocytes were recovered from 25 (74%) of the follicles, but two of these were lost during the staining procedure. Sixteen oocytes had condensed chromatin; five oocytes had a fluorescent nucleus and two oocytes had abnormal configurations. An adequate number of granulosa cells was obtained from 27 (79%) of the follicles. Apoptosis was detected in 58% of follicles ranging from 3 to 10 mm in diameter and 40% of follicles 11–20 mm in diameter. Approximately equivalent percentages of follicles 11–20 mm in diameter (50%) and 31–60 mm in diameter (40%) showed DNA smearing, whereas this was observed in only 17% of follicles 3–10 mm in diameter.

Forty-four percent of the oocytes retrieved had a compact cumulus mass, 52% had an expanded cumulus mass and 4% were denuded. Of the oocytes with compact cumuli, 50% had a fluorescent nucleus and 40% had compacted chromatin. All of the oocytes with expanded cumuli had

Table 2. Comparison of features of small (3–20 mm) and large (31–60 mm) equine follicles in Expt 2

	Percentage of follicles with each feature	
	Group A (3–20 mm)	Group B (31–60 mm)
Compact cumulus oophorus	37	80
Expanded cumulus oophorus	58	20
Fluorescent nucleus	24	20
Condensed chromatin	71	60
Granulosa cell apoptosis	50	0

compacted chromatin. Ten percent of follicles containing oocytes with compacted cumuli were apoptotic, and 60% of follicles containing oocytes with expanded cumuli had signs of apoptosis. In follicles containing oocytes with the fluorescent nucleus configuration, no apoptosis was detected, but 43% of follicles containing oocytes with compact chromatin were apoptotic. In group A (3–20 mm diameter), 55% of follicles containing an oocyte with compacted chromatin were apoptotic, whereas in group B (31–60 mm diameter), there were no oocytes with compacted chromatin in apoptotic follicles. A comparison between group A and group B is shown (Table 2).

Discussion

Studies on atresia in equine follicle populations have been conducted using histological material to classify follicles as growing or regressing (Kenney *et al.*, 1979; Driancourt *et al.*, 1982a). In the present study, a technique was used to identify apoptosis, a form of physiological cell death. The highest incidence of apoptosis was found in follicles 6–10 mm in diameter, indicating that in this size group there is considerable selection for survival or regression. Fewer larger follicles (> 15 mm diameter) were apoptotic and no follicles > 30 mm were apoptotic, indicating that once the follicles have reached this size, they either ovulate, or die by means other than apoptosis. Tilly (1991) reported that preovulatory hen follicles are resistant to apoptosis, and the results of the present study indicate that equine follicles are also resistant to this form of cell death. Hinrichs (1991) reported that the viability of mare follicles increased with increasing follicle size; the percentage of atretic follicles was 76, 81, 58 and 17% for follicles < 4, 5–9, 10–19 and > 20 mm in diameter, respectively. Similar results were obtained in the present study: the percentages of apoptotic follicles in similar size-differentiated groups were 49, 51, 45 and 21%, respectively. However, it is possible that the method used in the present study did not detect apoptosis in very atretic follicles, because the granulosa cells had degenerated and it was not possible to extract sufficient DNA. Kenney *et al.* (1979) reported that in follicles with mild atresia (detected histologically), atretic granulosa cells were not distributed uniformly, but were observed in focal points of atresia within areas of granulosa cells with normal morphology. This adds uncertainty to the reliability of the histological method for determining the presence of atresia, in which only a small section of the follicle wall is examined. In the present study, most of the granulosa cells in each follicle were examined for apoptosis.

The presence of a range of sheared DNA fragments of different sizes in many follicles in the present study may indicate the presence of necrosis, although DNA smearing is not a definitive way of detecting necrosis. Therefore, the collection, storage, handling and extraction procedures might have led to artefactual results. Herrmann *et al.* (1994) suggested that DNA smearing of DNA may depend on the method of DNA extraction used. In the present study it was observed that DNA extracted from large follicles smeared more than DNA from small follicles. Ethidium bromide staining is a less sensitive technique for detecting laddering than smearing compared with 3′-end-

labelling. This could have biased the results, if primarily small follicles had been examined with 3'-end-labelling. However, there was no clear pattern as to which size range of follicles would yield sufficient DNA for ethidium bromide staining. The only pattern observed was that follicles with a thick layer of granulosa cells, regardless of follicle size, contained a sufficient amount of DNA for ethidium bromide staining.

Granulosa cells from five follicles were kept at room temperature in follicular fluid for 0, 1, 2, 3 and 5 h before centrifugation and snap freezing to study the effect of the time period between death of the mares and sample processing (maximum 6 h). Agarose gel electrophoresis showed no differences in the degree of DNA smearing in any of these treatments. This finding indicates that it is unlikely that the smearing occurred as a post-mortem artefact due to holding time, but may reflect the degree of breakdown of DNA into a range of different sizes *in vivo*. Among the species commonly studied, the horse is the only one that has such large follicles. The granulosa cell layer is thick and unvascularized and the cells may undergo some degenerative changes, which could potentially appear as DNA smearing and possibly necrosis. If the DNA smearing observed in the present study did occur *in vivo*, it can be concluded that the differences between the two modes of cell death are ill-defined and that, after apoptosis, cells may undergo secondary necrosis (Zamzami *et al.*, 1997). Jolly *et al.* (1997) observed necrotic granulosa cells in late stages of atresia in ewes. Recent reports (Columbano, 1995; Raffray and Cohen, 1997) have questioned whether apoptosis and necrosis are separate death pathways, or if they overlap. Necrotic cell morphologies may be accompanied by the internucleosomal DNA cleavage that is thought to be characteristic of apoptosis, or apoptosis may be accompanied by 'necrotic' DNA smearing (Raffray and Cohen, 1997).

Driancourt *et al.* (1982b) reported that just before ovulation all follicles > 10 mm in diameter were atretic apart from the preovulatory follicle. In the present study (Expt 2), only 10% of follicles between 10 and 20 mm in diameter were classified as healthy. Apoptosis was detected in 40% of the follicles (10–20 mm diameter) and 50% showed DNA smearing. In preovulatory pig follicles, apoptosis was observed primarily in follicles < 3 mm in diameter (small follicles), and the smallest number of apoptotic cells was seen in follicles > 5 mm in diameter (large follicles) (Garrett and Guthrie, 1997). The present study supports these findings, as apoptosis was observed only in equine follicles < 20 mm in diameter (small follicles) in mares with a large follicle present on the ovary.

In the present study, the chromatin configuration did not tend to differ in oocytes from large and small follicles. However, apoptotic follicles had no oocytes with fluorescent nuclei but did contain some oocytes that had entered germinal vesicle breakdown (GVBD) and also some degenerative oocytes. In contrast, non-apoptotic follicles contained oocytes with fluorescent nuclei but no oocytes undergoing GVBD. Hinrichs and Williams (1997) reported that the incidence of the condensed chromatin configuration was lower in non-atretic follicles. They also reported that fluorescent nuclei were not observed in very atretic follicles and that expanded cumulus oophorus oocytes were likely to derive from atretic follicles. These findings are consistent with the results of the present study and indicate that the follicles potentially selected for ovulation are not undergoing apoptosis and that apoptosis is present in non-ovulatory follicles that are destined for regression. Whether apoptosis is present at the time of selection or if it appears at a later stage remains to be elucidated.

This method of evaluation of follicular status only allows a follicle to be studied at one particular point in time. Cells undergoing apoptosis can become undetectable within 24 h (Kerr *et al.*, 1972). Therefore, it is not known whether the changes observed occurred at an early or late stage in apoptosis, only that apoptosis is present. Furthermore, apoptosis and mitosis can be present in the same follicle, and therefore atresia is determined by whichever predominates.

This is the first report to describe apoptotic cell death in equine granulosa cells. Apoptosis, as a marker of cell death, enables investigation of the growth and selection of follicles, and can be used to correlate the health of granulosa cells with oocyte quality.

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Method for isolating preantral follicles from mare ovaries

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The aims of this study were to evaluate the use of collagenase treatment to isolate preantral follicles from mare ovaries and to assess the effect of this treatment on follicular morphology. Intact mare ovaries were chopped into pieces, incubated individually with 1, 3 or 5 mg collagenase (type 1A) ml^{-1} in a shaking waterbath at 37°C for up to 2 h and passed through a series of stainless steel filters with pore size 50–300 μm to remove large clumps and stromal cells. The samples were prepared for histological analysis and sections were examined by light microscopy. Isolated follicles and oocytes were measured and the quality of the follicles was assessed by microscopic examination. Very few intact preantral follicles were isolated after 30 min incubation with 1, 3 or 5 mg collagenase ml^{-1}. After 60 and 90 min incubations, between eight and 71 intact preantral follicles were isolated with 3 or 5 mg collagenase ml^{-1}, whereas very few were isolated after incubation with 1 mg collagenase ml^{-1}. The number of intact follicles isolated after incubation with either 3 or 5 mg collagenase ml^{-1} was not significantly different. The quality of the isolated follicles decreased with increasing incubation time and no intact follicles were observed after 2 h of incubation. Preantral follicles 60–300 μm in diameter were isolated from ovaries after treatment with either 3 or 5 mg collagenase ml^{-1}. Most of the follicles isolated were 90–150 μm in diameter. This study indicates that equine preantral follicles can be isolated from equine ovaries using collagenase and that collagenase does not have a deleterious effect on follicle morphology when used at the appropriate concentration for a minimum period. However, oocyte quality and follicle viability remain to be determined.

Introduction

Assisted reproduction techniques developed for other species have been difficult to use in mares. For example, there are no consistently successful methods for obtaining large numbers of immature oocytes from live mares, for inducing maturation of the oocytes or for fertilization *in vitro*. The major limitation to successful IVF in mares is the difficulty in obtaining large numbers of oocytes for fertilization (Hinrichs, 1992; Boyle, 1993). Immature oocytes have been obtained from equine ovaries by aspiration, slicing or scraping of ovaries collected at post-mortem (Okolski *et al.*, 1991; Choi *et al.*, 1993; Hinrichs *et al.*, 1993), by follicular puncture through a flank incision of live mares (Palmer *et al.*, 1987; Hinrichs *et al.*, 1990) and by ultrasound-guided transvaginal puncture (Cook *et al.*, 1993; Duchamp *et al.*, 1995; Kanitz *et al.*, 1995). However, recovery rates are low. Approximately four to seven oocytes are obtained typically from a single animal at post-mortem (Hinrichs, 1991; Okolski *et al.*, 1993; Brinsko *et al.*, 1995; Del Campo, 1995), although Zhang *et al.* (1989) obtained five to ten oocytes per ovary. It is not usually possible to obtain large numbers of fresh equine ovaries at post-mortem. The mean recovery rate per aspiration procedure performed on live mares is less than one oocyte per week (Duchamp *et al.*, 1995) or 3.4 oocytes per mare when the aspirations are performed

less frequently (Kanitz *et al.*, 1995). Furthermore, 70–75% of the oocytes recovered after needle aspiration do not have a complete cumulus mass (Hinrichs, 1991; Kanitz *et al.*, 1995). The highest oocyte recovery rates from live mares have been obtained by repeated ultrasound-guided oocyte retrieval during early pregnancy. Meintjes *et al.* (1995) used this technique to perform a mean of 7.6 aspirations per mare between days 21 and 150 of pregnancy and obtained a mean of 18.9 oocytes per mare. However, this technique is time-consuming and does not produce enough high quality oocytes to enable the development of reproducible IVF techniques.

Studies in other species have investigated the use of the preantral follicles in the ovaries, most of which become atretic during the life of the animal. These follicles are a rich source of biological and genetic material that could be used in assisted reproduction techniques. Furthermore, the development of freezing techniques that enable subsequent production of oocytes from frozen primary follicles (Carroll *et al.*, 1990) means that genetic material can be conserved for many years. Preantral follicles have been isolated and cultured successfully from laboratory animals (Roy and Greenwald, 1989; Carroll *et al.*, 1990; Eppig and O'Brien, 1996), dogs (Durrant *et al.*, 1993), pigs (Hirao *et al.*, 1994), cattle (Nuttinck *et al.*, 1993) and humans (Roy and Treacy, 1993). Eppig and Schroeder (1989) successfully produced large numbers of meiotically competent mouse oocytes, which were fertilized *in vitro*, transferred to a female mouse and resulted in live young.

There are approximately 35 000 primordial follicles in the ovary of a young mare (Driancourt *et al.*, 1982). Equine ovaries are fibrous and contain few oocytes compared with other species, making it more difficult to isolate preantral follicles from horses than from other species. The aim of the present study was to extract preantral follicles from horse ovaries and to assess follicular morphology.

Materials and Methods

Collection of animal tissues

Mare ovaries were obtained either by ovariectomy or at post-mortem. Pairs of ovaries from each of ten mares aged 2–4 years were used.

Optimization of collagenase concentration and incubation time

The first experiment was performed to determine the optimum collagenase concentration and incubation time. The corpora lutea and large follicles were removed from eight ovaries and the ovaries were cut into approximately 3 mm^2 pieces. Pieces from individual ovaries were incubated with either 1, 3 or 5 mg collagenase ml^{-1} (type 1A; Sigma, Poole) in 40 ml Hepes buffered medium M199 (Sigma, Poole) containing 3 mg BSA fraction V ml^{-1} (Sigma, Poole) in a 50 ml disposable plastic test tube (Costar, Cambridge, MA). The tubes were placed in a shaking waterbath at 37°C. Samples were taken from each tube at 30 min intervals for up to 2 h. Two ovaries were treated with 1 mg collagenase ml^{-1} and 3 or 5 mg collagenase ml^{-1} treatments were each performed on three ovaries. After each 30 min period, the pieces were pipetted up and down using a Pasteur pipette and the medium was passed through a 300 µm steel mesh filter. The remaining pieces were transferred back to the tubes and were incubated for a further 30 min. The material that passed through the 300 µm filter was washed with 15 ml M199 and 10% (v/v) fetal calf serum to inactivate the collagenase. The filtrate was passed through a series of graded filters with mesh sizes 200, 150, 100 and 50 µm. At each stage, the material retained by the filter was transferred to an Eppendorf microtube and suspended in a collagen gel solution (Eppig and Telfer, 1992) to be processed for histological examination. The final filtrate was centrifuged at 1000 *g* for 5 min and the resulting pellet was suspended in collagen gel for histological processing.

Assessment of follicle yield per ovary

The second experiment was performed to determine the number of follicles isolated from

individual intact ovaries. Twelve ovaries were cut into pieces as decribed earlier and the pieces were incubated separately in 5 mg collagenase ml^{-1}. Six ovaries were incubated for 60 min and six were incubated for 90 min. At the end of each incubation period, the samples were processed as described earlier.

Histology

The collagen gel droplets containing the material from each stage of the collagenase digestion were fixed with Bouin's fixative for 24 h and dehydrated in a graded series of alcohol up to absolute alcohol, for 60 min at each stage. The collagen drops were cleared in cedar wood oil, transferred to toluene and embedded in paraffin wax. Serial sections were cut at 7 μm intervals and mounted on gelatine-coated glass slides. The sections were stained with haematoxylin and eosin.

Assessment of follicles

The slides from the ovarian treatments were examined at × 20 and × 40 magnification. In each section, the number of preantral follicles was counted and the integrity of the granulosa cell layer and of the oocyte was recorded. Intact follicles were defined as follicles in which the oocyte was surrounded by complete layers of granulosa cells. Oocyte quality was assessed by observation of the cytoplasmic shape and the presence of a germinal vesicle. Degenerate follicles were defined as follicles in which one or more of the following features was observed: (i) the oocyte was surrounded by incomplete layers of granulosa cells; (ii) 20% of the granulosa cells had pycnotic nuclei; or (iii) a degenerate oocyte was present. These criteria have been used to assess isolated bovine follicles (van den Hurk *et al.*, 1998). The diameters of the follicles and oocytes were measured at × 40 magnification with an ocular micrometer.

Statistical analysis

A paired *t* test using EXCEL was used to compare the number of follicles isolated from ovaries after incubation with collagenase for different time periods. A comparison of the total number of follicles isolated from individual ovaries was performed using an unpaired *t* test.

Results

Optimization of collagenase concentration and incubation time

Identification of follicles in the histological sections was difficult because the samples were contaminated with clumps of stromal cells, despite the fact that a small filter was used during sample preparation to filter out these cells. The mean total number of follicles isolated from ovaries (intact and degenerate follicles) for each collagenase concentration and incubation time is shown (Table 1). These results indicate that incubation with 1 mg collagenase ml^{-1} is an ineffective method for isolating follicles. The mean number of intact follicles isolated at different collagenase concentrations at 30 min intervals during a 2 h incubation is shown (Fig. 1). The number of intact follicles isolated using 1 mg collagenase ml^{-1} was small, but at 3 and 5 mg collagenase ml^{-1} larger numbers of intact follicles were isolated after 60 and 90 min incubation. The number of intact follicles isolated after 60 and 90 min varied among samples (range 15–71) but there was no significant difference between the number of follicles isolated with either 3 or 5 mg collagenase ml^{-1} (Fig. 1). No intact preantral follicles were detected after 2 h incubation with either 3 or 5 mg collagenase ml^{-1}, although degenerate follicles were observed in samples incubated with 1, 3 or 5 mg collagenase ml^{-1} for 2 h (Fig. 2). The number of degenerate follicles increased with time in all the treatment groups and a significantly greater number was observed after 90 or 120 min incubation with 3 or 5 mg collagenase ml^{-1} than with 1 mg collagenase ml^{-1} ($P < 0.05$) (Fig. 2).

Table 1. Number of preantral follicles (intact and degenerate) isolated from equine ovaries after 30, 60, 90 and 120 min incubation

	Collagenase (mg ml^{-1})		
Incubation time (min)	1	3	5
30	4	13 ± 1.1	14 ± 2.9
60	7	42 ± 3.2	47 ± 5.1
90	6	60 ± 4.2	54 ± 6.7
120	6	25 ± 1.6	30 ± 2.2

Values are mean ± SEM for 3 mg collagenase ml^{-1} (n = 3) and 5 mg collagenase ml^{-1} (n = 3), and mean for 1 mg collagenase ml^{-1} (n = 2).

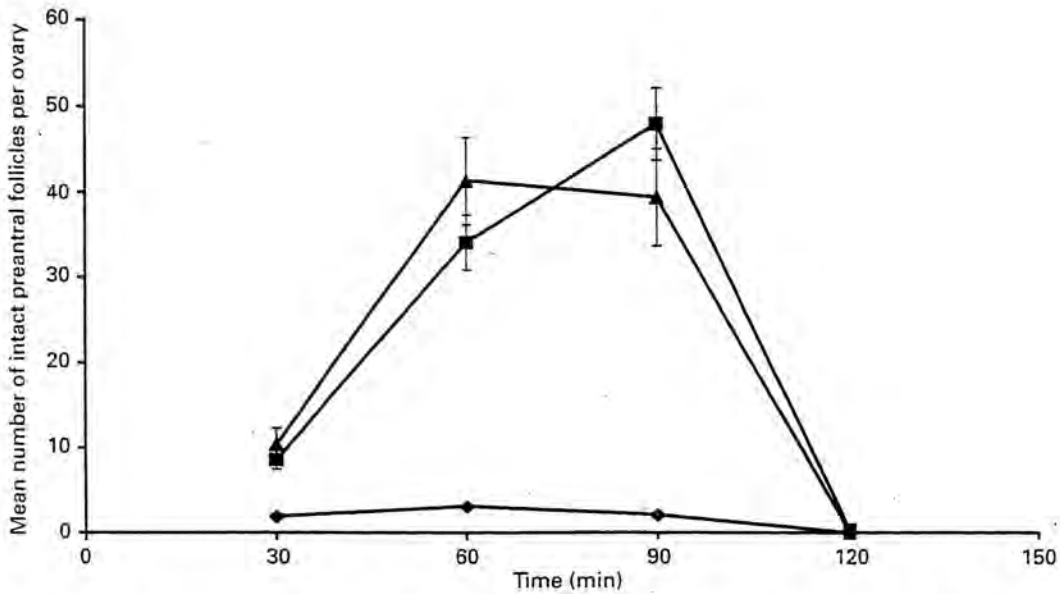

Fig. 1. Mean ± SEM number of intact preantral follicles isolated per equine ovary after 30, 60, 90 and 120 min incubation with three concentrations of collagenase: (◆) 1 mg collagenase ml^{-1} (n = 2); (■) 3 mg collagenase ml^{-1} (n = 3); and (▲) 5 mg collagenase ml^{-1} (n = 3).

Assessment of follicle yields per ovary

The total number of follicles obtained per ovary ranged from ten to 58 after 60 min incubation and from 30 to 101 after 90 min incubation (Table 2). The mean total number of follicles (intact and degenerate) isolated per ovary was 33 ± 7.3 after 60 min and 57 ± 11.3 after 90 min ($P < 0.05$). The number of intact follicles isolated after 90 min was not significantly greater than the number isolated after 60 min incubation. However, the number of degenerate follicles present after 90 min incubation (17 ± 3.3) was significantly greater than the number obtained after 60 min incubation (7 ± 1.1) ($P < 0.05$). No follicles > 300 μm in diameter were isolated and 70% of all the follicles isolated were between 90 and 150 μm in diameter.

Table 2. Total number of follicles and number of degenerate follicles isolated from individual equine ovaries

	60 min incubation		90 min incubation	
	Total number of follicles isolated per ovary	Number of degenerate follicles isolated per ovary	Total number of follicles isolated per ovary	Number of degenerate follicles isolated per ovary
	10	2	62	21
	36	6	36	8
	26	7	30	10
	20	5	37	15
	58	15	75	20
	48	9	101	30
Mean ± SEM	33 ± 7.3	7 ± 1.1	57 ± 11.3	17 ± 3.3

Ovaries were incubated with 5 mg collagenase ml^{-1} for either 60 ($n = 6$) or 90 ($n = 6$) min.

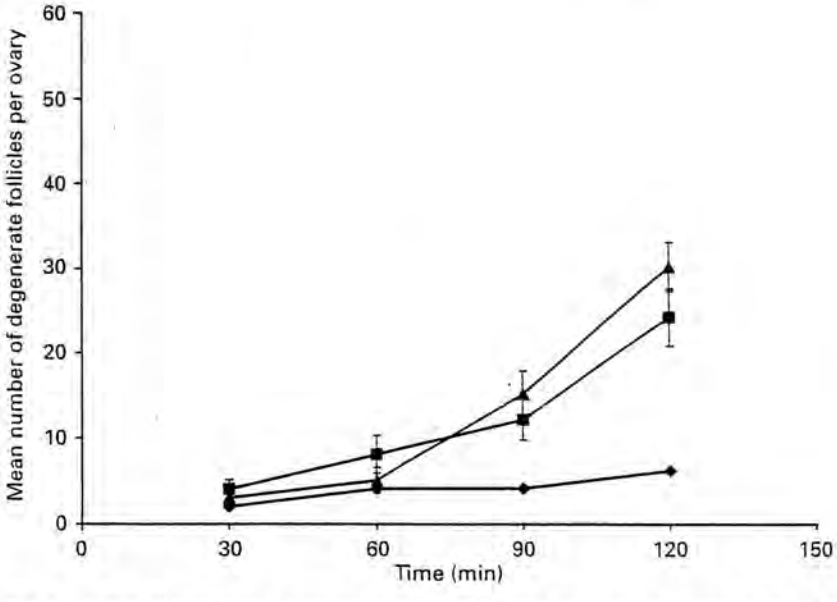

Fig. 2. Mean ± SEM number of degenerate preantral follicles isolated per equine ovary after 30, 60, 90 and 120 min incubation with three concentrations of collagenase: (◆) 1 mg collagenase ml^{-1} ($n = 2$); (■) 3 mg collagenase ml^{-1} ($n = 3$); and (▲) 5 mg collagenase ml^{-1} ($n = 3$). Degenerate follicles were defined as those in which one or more of these features was observed: (i) the oocyte was surrounded by incomplete layers of granulosa cells; (ii) 20% of the granulosa cells had pycnotic nuclei; or (iii) a degenerate oocyte was present.

Discussion

The number of preantral follicles in the ovaries of an animal varies with its age and physiological status. Studies in mares have indicated that there are approximately 35 000 primordial follicles (Driancourt *et al.*, 1982) and 1000 preantral follicles (Szlachta and Tischner, 1998) in the ovaries of young mares. Isolation and maturation of oocytes at this stage of development would represent a major advance in the production of equine embryos *in vitro*.

Several methods have been developed for isolating preantral follicles from rodent ovaries, which typically involve tissue disaggregation to obtain the preantral follicles. An enzymatic dissociation procedure involving collagenase has been used for isolating follicles from the ovaries of rabbits (Nicosia *et al.*, 1975), mice (Eppig and Schroeder, 1989; Torrance *et al.*, 1989), rats (Roy and Greenwald, 1996) and hamsters (Roy and Greenwald, 1985, 1989). These methods have enabled large numbers of early preantral follicles to be collected and cultured *in vitro* to a stage at which the oocytes can resume meiotic maturation and be fertilized.

Isolation of large numbers of preantral follicles from domestic species has been more difficult (Telfer, 1996, 1998). The ovaries of most domestic species are fibrous and therefore techniques used for laboratory species cannot always be applied. Isolation of follicles from the equine ovary is particularly difficult as there are large quantities of stromal tissue and relatively few immature follicles compared with the numbers found in other species. This study has demonstrated that preantral follicles may be obtained from the ovaries by incubation with 3 or 5 mg collagenase ml^{-1}. The total number of intact follicles isolated from an individual ovary did not exceed 80. This number is low compared with other species and much lower than was expected on the basis of histological studies. For example, Szlatchna and Tischner (1998) observed up to 85 preantral follicles in a single section of an equine ovary. The low number of preantral follicles isolated in this study may be due to an inefficient isolation technique and degradation of the follicles by collagenase. Most of the intact follicles (70%) were between 90 and 150 μm in diameter, indicating that they were early preantral follicles. These follicles would take months to mature; thus a prolonged culture system would be required to grow this population *in vitro*. No follicles > 300 μm in diameter were isolated, indicating that the filters successfully excluded larger clumps of follicles.

The quality of the oocytes obtained after collagenase treatment was not evaluated fully in this study. Although the oocytes in the intact follicles appeared to be viable, whether they would grow and develop *in vitro* is uncertain. Cattle oocytes are sensitive to collagenase treatment and this enzyme cannot be used for isolating preantral follicles from bovine ovaries (Wandji *et al.*, 1996). Further studies are required to determine whether equine oocytes are also sensitive to collagenase and whether follicles isolated using collagenase treatment are capable of growing and developing *in vitro*. However, a more effective method for separating follicles from stromal cells is required before the sensitivity of equine oocytes to collagenase can be evaluated.

In the present study, the number of follicles isolated varied among samples, probably because of variations in the age of the mares and in the effectiveness of the isolation procedure. This study was based on histological evidence and the number of follicles detected here does not reflect the total number that could potentially be isolated as intact whole follciles. It is unlikely that all the follicles detected by this method could be recovered intact due to the problems of stromal cell contamination.

The main findings of this study were: (i) intact preantral follicles can be isolated from mare ovaries with collagenase; and (ii) the number of degenerate follicles increases with increasing incubation time with collagenase. Improved techniques for removing stromal cells from samples are required before an effective method for isolating preantral follicles from mare ovaries can be developed.

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REVIEW

Infertility in the Mare

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Mares have a pregnancy rate lower than that of any other domestic species. Poor management accounts for many pregnancy failures, but this review will be confined to the pathological causes of infertility.

Ovary

Special Features of Normal Equine Ovaries

The mare ovary is kidney bean-shaped with a depression, the ovulation fossa, on the free (ventral) border. The medullary or vascular zone, unlike that of other species, is superficial and the cortical zone, which contains the follicles, is partly in the interior of the ovary. The cortex reaches the surface only at the ovulation fossa, which in the mare is the only site at which ovulation can occur (Witherspoon, 1975). The corpus luteum of the mare has two recognized morphologies, both of which are normal. A central blood clot develops after ovulation in some (*c.* 50%) corpora lutea but not in others (Ginther, 1986). The clot remains throughout the functional lifespan of the corpus luteum but becomes smaller and increasingly organized. During pregnancy (between days 40 and 180, approximately) secondary corpora lutea develop after ovulation or luteinization of anovulatory follicles. This is probably due to the effect of equine chorionic gonadotrophin from the endometrial cups. The numbers of these supplementary corpora lutea increase from an average of 2·8 at day 70 to 10·2 at day 140 (Martin *et al.*, 1989). The foeto-placental unit takes over as the primary source of progestogens from about day 150 of pregnancy.

Adrenocortical nodules are common near the ovary in the mare. They have been reported in 59% of 271 mares and were described as yellow, 1–2 mm in diameter and arranged in grapelike clusters in the connective tissue over the ovary (Ono *et al.*, 1969). The functional importance of these structures is not known.

*Hypoplasia**

Affected mares have normal external genitalia but may be infertile. Cyclical ovarian activity may be abnormal or absent, the ovaries consisting primarily of undifferentiated stroma. The uterus is flaccid, with an underdeveloped

* Results in small ovaries.

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endometrium, and circulating concentrations of luteinizing hormone (LH) are elevated because of the absence of "negative feedback" from ovarian steroids.

By far the commonest cause is 63X or 63XO gonadal dysgenesis, an aneuploid defect of the sex chromosomes (Chandley *et al.*, 1975; Hughes *et al.*, 1975) analogous to Turner's syndrome in women. Approximately 15% of affected mares have a mosaic karyotype with either 63X/64XX or 63X/64XY (Bowling *et al.*, 1987). Mares with 63X chromosome complement tend to be of small stature and are invariably sterile. In one study pregnancy was established in progestogen-supplemented 63XO mares by embryo transfer (Hinrichs *et al.*, 1989b), but other workers have failed to transfer embryos successfully to such animals (Pashen *et al.*, 1989).

*Atrophy**

Ovarian atrophy resulting in anoestrus can occur in mares debilitated from chronic disease, stress, parasitism or emaciation, or treated with reproductive steroids. The ovaries of a small proportion of mares reach senescence by 17 to 25 years of age (Wesson and Ginther, 1981) and Vanderwall and Woods (1991) showed that by 24 years of age 37% do not ovulate during the ovulatory season.

Ovarian Neoplasia†

Granulosa cell tumour. This is the most common ovarian neoplasm of the mare (2·5% of all equine neoplasms; Sunberg, 1977). The tumour, which is of sex cord origin, is usually unilateral and multicystic (Fig. 1). The mean age of affected mares is 10·6 years, with a range of 2–20 years (Meagher *et al.*, 1977). These tumours are frequently accompanied by endocrine disturbances such as anoestrus, continuous oestrus or stallion-like behaviour. The latter is associated with high concentrations of circulating testosterone, but the other types of behaviour are not obviously linked to reproductive hormone concentrations in the serum (Stabenfeldt *et al.*, 1979). Although oestradiol concentrations may be slightly elevated in mares with granulosa cell tumours, the high concentrations characteristic of the human tumour (Besch *et al.*, 1966) are not observed. Moreover, in women the tumour tends to metastasize (Kalavathi, 1971), whereas Meagher *et al.* (1977) reported that only one such tumour metastasized in 78 affected mares. Palpation of the ovaries *per rectum* generally reveals one large ovary, in which the ovulation fossa is obliterated, and a small inactive contralateral ovary, but the ultrasonographical appearance is not sufficiently characteristic to be diagnostic (Hinrichs and Hunt, 1990). Granulosa cell tumours in mares express mRNA for inhibin (Piquette *et al.*, 1990) and it has therefore been suggested that the contralateral ovarian atrophy results from high concentrations of circulating inhibin. Alternatively, the hypothalamic-pituitary axis may be suppressed by negative feedback from

* Results in small ovaries.
† Results in enlarged ovaries.

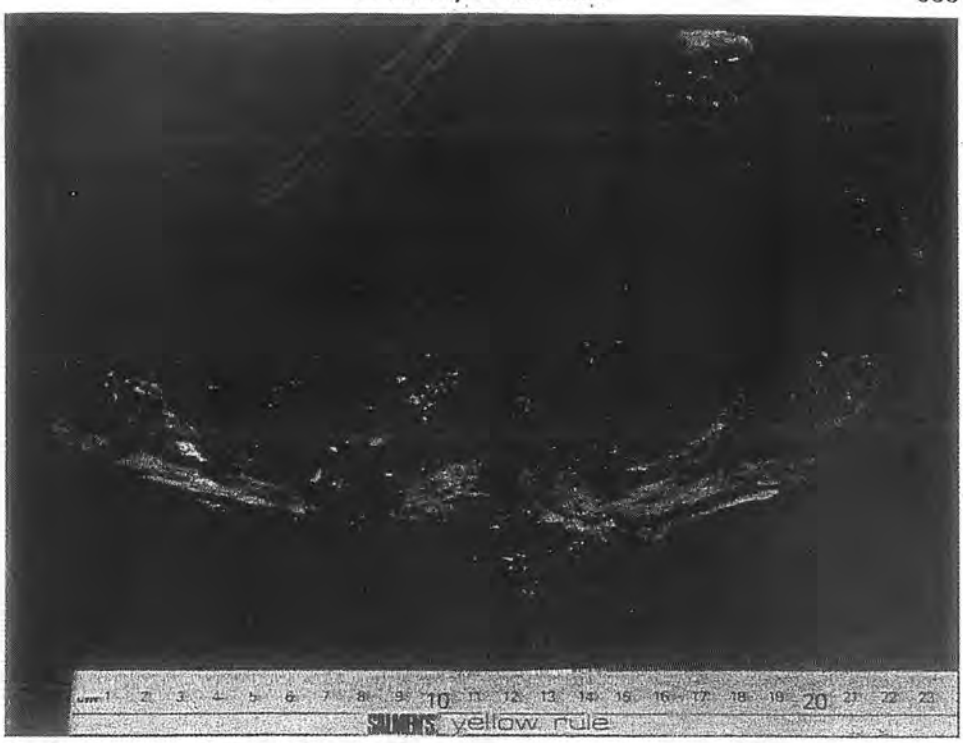

Fig. 1. Granulosa cell tumour from a mare.

high circulating concentrations of ovarian steroids. Occasionally a granulosa cell tumour may be present in a mare with a functional contralateral ovary (Hinrichs *et al.*, 1990; McCue *et al.*, 1991) and two mares have been reported as maintaining their pregnancies with a granulosa cell tumour on one ovary (Meagher *et al.*, 1977). After removal of a neoplastic ovary, the other ovary usually resumes cyclic activity between 3 and 12 months later (Stabenfeldt *et al.*, 1979).

The majority of granulosa cell tumours have a diameter of 10 to 20 cm and are predominantly cystic with yellow stroma. The cysts are lined by granulosa cells surrounded by an irregular layer of thecal or stromal cells (Fig. 2). Granulosa cells are also found in trabecular cords or in solid masses according to Stabenfeldt *et al.* (1979), who also reported the presence of large "Leydig-like" polyhedral cells in the stromal tissue of some tumours. McEntee (1990a) suggested that these cells resembled luteal cells more closely than testicular interstitial cells. Their presence in tumours was associated with increased circulating concentrations of testosterone (Stabenfeldt *et al.*, 1979). Because of the significant part played by thecal cells in the formation of these neoplasms, Stabenfeldt *et al.* (1979) suggested that they should more correctly be termed granulosa-thecal cell tumours.

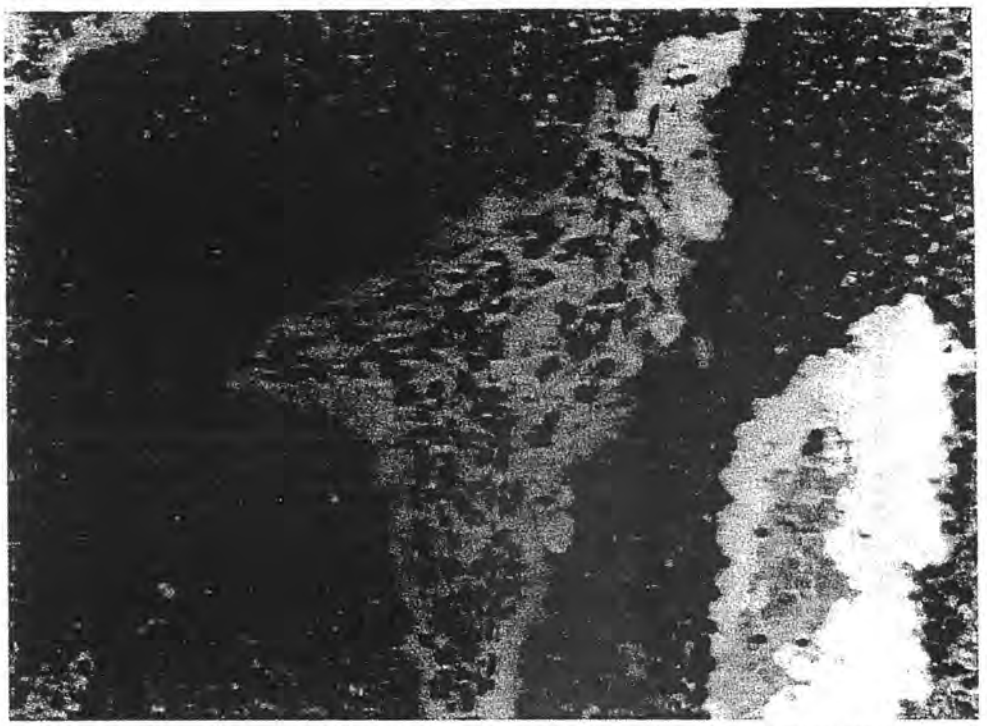

Fig. 2. Granulosa cell tumour. Solid cords of neoplastic granulosa cells with cystic spaces containing cell debris. A small cyst lined by granulosa cells is visible. HE. High power.

Cystadenoma and cystadenocarcinoma. Cystadenoma, a primary epithelial neoplasm which is usually benign, is not as a rule secretory, although in two cases elevated concentrations of circulating testosterone were reported (Hughes *et al.*, 1980; Hinrichs *et al.*, 1989a). The activity of the contralateral ovary is unaffected and the ovarian cycle is normal. Because of the origin of the neoplasm, the first palpable change in the ovary is loss of the ovulation fossa. The affected ovary usually comprises multiple large cysts containing clear yellow fluid (Hughes *et al.*, 1980; Held *et al.*, 1982) and lined by low cuboidal ciliated epithelium, the stroma consisting of dense connective tissue.

Adenocarcinomas have been reported in two mares, both of which were humanely destroyed because of multiple metastases (Morris *et al.*, 1985; Van Camp *et al.*, 1989).

Dysgerminoma. This rare malignant ovarian tumour, of germ cell origin, often causes chronic weight loss and abdominal discomfort due to abdominal metastases, and has been associated with hypertrophic osteopathy in mares (McLennan and Kelly, 1977; Meuten and Rendano, 1978). The tumour is cystic and comprises multiple lobules bounded by thick bands of connective tissue. The neoplastic cells are arranged in cords, sheets and alveoli of polyhedral cells, frequently undergoing mitosis. Necrotic foci are present,

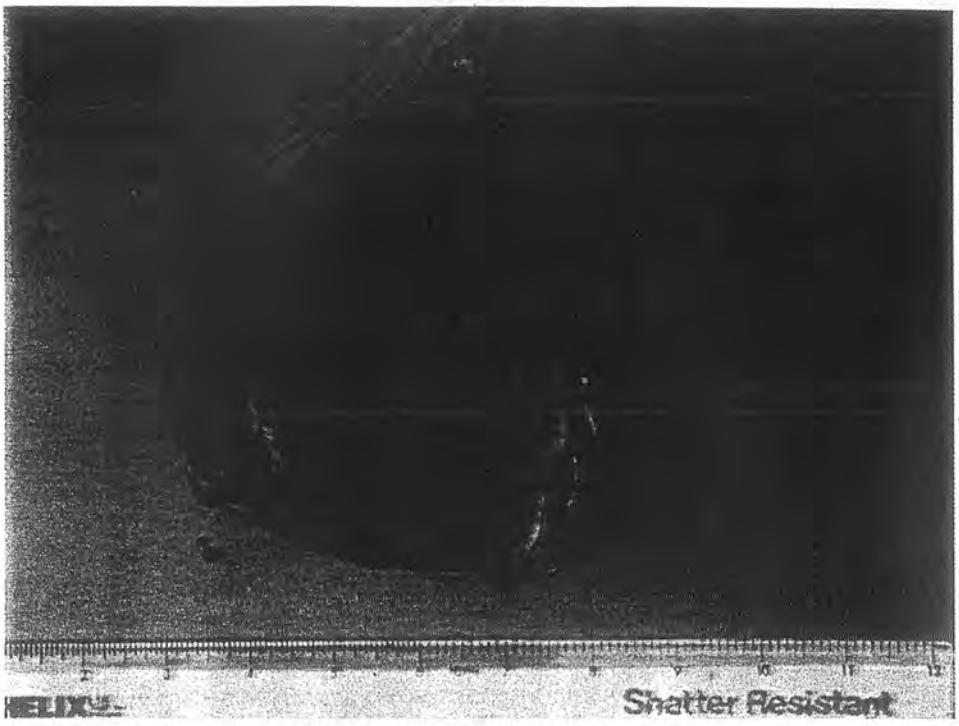

Fig. 3. Ovarian haematoma with no normal ovarian tissue remaining.

leaving spaces which may contain macrophages. Lymphocytic foci are rare.

Miscellaneous. Other tumours, which have been reported rarely, include melanoma, teratoma, lymphosarcoma and haemangioma.

Haematoma†

Haematomas, commonly found during the breeding season, result from excessive bleeding at ovulation which fills and distends the follicular cavity. They can be very large (>10 cm in diameter) but usually regress over two or three oestrous cycles, sometimes leaving areas of calcification. The mare will continue to have normal cycles (Hughes *et al.*, 1972). Occasionally, instead of regressing, a haematoma may destroy part or all of the ovary (Fig. 3).

Anovulatory Follicles†

This abnormality is frequently confused clinically with cystic ovaries. In autumn, mares often develop large anovulatory follicles (10–15 cm in diameter)

† Results in enlarged ovaries.

containing blood with a liquid to gelatinous consistency. These structures have been termed "autumn follicles" (Stangroom and Weevers, 1962), although they have been reported at other times of the year (Ginther and Pierson, 1989). The cause of these haemorrhagic follicles is unknown, but it has been suggested that their incidence in autumn is associated with declining gonadotrophin concentrations (Neely *et al.*, 1983).

Ovarian Abscess†

Abscesses are rare and generally only occur after needle aspiration of follicles.

Cysts Around the Ovary†

Cysts of mesonephric origin. These, usually called paraovarian or epoophoron cysts, are sometimes found cranial to the ovary and along the ovarian ligament (Osborne, 1987).

Cysts of paramesonephric origin. These can occur in the region of the ovulation fossa (O'Shea, 1978; Osborne, 1987).

Epithelial inclusion cysts. These, often called fossa cysts, originate in the ovulation fossa. The surface epithelium is disrupted at ovulation and becomes pinched off and embedded in the ovarian cortex (McEntee, 1990b). The cysts may become numerous in older mares and may eventually obstruct ovulation. In severe cases most of the ovary is destroyed.

Uterine Tube

The uterine tube of the mare expands cranially to form the infundibulum which covers the ovulation fossa at the time of ovulation. Some but not all unfertilized ova are retained in the uterine tubes, whereas fertilized ova are transferred to the uterus (van Niekerk and Gerneke, 1966; Wilson *et al.*, 1991). Globular masses which originate from the mucosa of the uterine tubes are also common in the distal ampulla (Onuma and Ohnami, 1975).

Significant abnormalities of the oviduct are rare in mares (McEntee, 1990c). Accessory oviducts may develop in the embryo at the end of the paramesonephric duct and persist as a cyst, or series of cysts, attached to the caudal part of the uterine tube. Archbald *et al.* (1974) suggested that large cysts could cause infertility.

Adhesions of the uterine tube to the ovary, uterus and mesovarial borders of the broad ligament, presumed to be secondary to salpingitis, are common in the mare (Henry and Vandeplassche, 1981), but do not normally cause occlusion.

Hydrosalpinx sometimes occurs as a result of external pressure from adhesions obstructing the lumen (McEntee, 1990c) or of segmental aplasia (Hughes, 1993).

Uterus

The mare's uterus, consisting of two horns and the body, has a lumen which is obliterated by the close apposition of the walls and prominent longitudinal folds. The latter extend caudally into the cervix and are composed of a longitudinal gland-free core of connective tissue covered on both sides by endometrium. During oestrus the folds become oedematous. The epithelial cells of the endometrium are columnar and ciliated. The lamina propria consists of the stratum compactum, which is about 1 mm thick and contains densely packed stromal cells and gland ducts, and the stratum spongiosum, which consists of loose connective tissue containing prominent branched and coiled glands lined by simple columnar epithelium.

The characteristic changes in the appearance of the uterus which occur at different stages of the oestrous cycle have been described by Kenney (1978). The luminal epithelium reaches its maximal height (30–40 μm) during early oestrus; this drops to 15–20 μm in late oestrus and early dioestrus and then increases through dioestrus and prooestrus. Neutrophils become marginated in blood vessels during oestrus but do not migrate through the stroma. During dioestrus the glands become highly coiled, whereas during oestrus they appear straight and well spaced. Some tissue oedema may be apparent during oestrus.

Postpartum uterine involution. Rapid uterine involution and an early postpartum fertile oestrus (the "foal heat") often result in the establishment of pregnancy within 1–2 weeks of parturition. Histologically, repair of the luminal epithelium and resorption of the microcaruncles is almost complete by 4–7 days postpartum (Gygax *et al.*, 1979; Bailey and Bristol, 1983). By day 4, endometrial gland dilation is no longer evident and glandular activity increases to day 12 postpartum (Bailey and Bristol, 1983). By day 14 postpartum the uterus has returned to its normal pregravid appearance (Gygax *et al.*, 1979).

Endometritis and Other Endometrial Abnormalities

Endometritis is probably the commonest cause of subfertility in mares. Some degree of acute endometritis is normal after coitus, parturition or veterinary gynaecological examination, but in a mare with a healthy uterus the infection will be eliminated within 72 hours (Peterson *et al.*, 1969). In some mares, however, uterine defence mechanisms fail to clear the infection because of a defect in the cellular or humoral immune clearance mechanisms (Watson *et al.*, 1987a; Watson and Stokes, 1990) or through inadequate uterine contractility (Troedsson and Liu, 1991). Such mares remain persistently infected and often respond poorly to treatment. Uterine infection that persists for more than 5 days after coitus will prevent successful pregnancy because of the embryotoxic environment and because of premature release of prostaglandin $F_{2\alpha}$, which will effect luteolysis (Watson *et al.*, 1987b). *Streptococcus zooepidemicus* is by far the most commonly isolated organism in mares with endometritis, followed by *Escherichia coli, Pseudomonas aeruginosa* and *Klebsiella pneumoniae.* Yeasts and fungi may also become established in the uterus, most frequently after

Fig. 4. Acute endometritis. Note the presence of many inflammatory cells in the stratum compactum and neutrophils migrating through the luminal epithelium. HE. High power.

antibiotic treatment, and may cause significant damage to the endometrium and other parts of the tract.

The disease is diagnosed by a combination of clinical and pathological techniques. Mares with persistent endometritis usually have a history of infertility and may or may not have a vulval discharge. Ultrasonographical examination of the uterus usually reveals the presence of luminal fluid, varying from "echogenic" (very cellular) to "clear" (fewer cells). Culture of endometrial material, collected aseptically with a double guarded swab, will frequently yield a heavy, pure growth of a potential pathogen. However, this should not be considered diagnostic in the absence of clinical signs, as accidental bacterial contamination of endometrial swabs is relatively common (Kenney, 1978). Cytological examination of an endometrial scraping or of luminal fluid will show the presence of many neutrophils (Knudsen, 1964; Waelchli *et al.*, 1988). Correctly prepared smears from normal mares rarely contain neutrophils unless collected shortly after copulation, foaling or uterine lavage. Histological examination of the endometrium of mares with acute endometritis will show neutrophils, usually in the stratum compactum and between luminal epithelial cells (Fig. 4). Neutrophils may sometimes also be found in the stratum spongiosum and in the gland lumina. In long-standing cases there will be infiltration of lymphocytes and plasma cells. Infiltration of eosinophils has

been associated with endometritis caused by an influx of air into the uterus from the vagina (Slusher *et al.*, 1984) and, infrequently, with fungal endometritis (Hurtgen and Cummings, 1982). Chronic inflammatory cell infiltrates most commonly occur in the stratum compactum. In the stratum spongiosum, infiltration most commonly takes the form of discrete foci of mononuclear cells, most of which are T lymphocytes and plasma cells (Watson and Stokes, 1988; Watson and Dixon, 1993).

Interpretation of endometrial biopsy findings has been thoroughly reported by Kenney (1978). The biopsy procedure may produce artefacts, which include haemorrhage, oedema, intussusception of glands and denuding of surface epithelium. Evaluation of the sample should take account of whether sufficient tissue has been collected (>1 linear cm), the histological structure related to the stage of cycle, the inflammatory response, and the degree of chronic irreversible changes. Under good breeding management, the likelihood of a mare carrying a foal to term is related not only to the degree of endometrial fibrosis but also to the duration of the barren period. Thus, Doig *et al.* (1981) found that mares that had been barren for only one year had twice the foaling rates of similar mares that had been barren for $\geqslant 2$ years.

Pathological changes within the endometria of mares have been categorized as I, IIA, IIB and III, depending on their degree and distribution (Doig *et al.*, 1981). In category I there are either no changes or they are mild and sparsely scattered. In category IIA the changes are slight to moderate and scattered. Changes are widespread and moderately severe in Category IIB and in Category III the changes are widespread, diffuse and severe.

A mare should never be condemned on the basis of one biopsy. Although the pathological changes tend to be uniform (Bergman and Kenney, 1975) focal lesions can occur (Gross and LeBlanc, 1984) and therefore it is sometimes necessary to collect endometrial biopsies from several sites.

As mares age, chronic irreversible changes occur within the endometrium. These are classed as normal if they fall within acceptable limits but, depending on their severity, they may significantly decrease the ability of a mare to carry a foal to term (Kenney 1978; Kenney and Doig, 1986). Such changes are of the following three basic types.

Endometrial fibrosis. When widespread, endometrial fibrosis is one of the main factors that lead to placental insufficiency and foetal loss. Collagen is most commonly deposited around the glands (Fig. 5) or under the basement membrane of the luminal epithelium. The first sign of periglandular fibrosis is loss of randomization of the endometrial stromal cells. Individual gland branches may be affected or several branches may be surrounded to form a "nest". The severity of fibrosis is classified by the number of periglandular layers (2–3 in mild cases to >10 in severe cases) and the number of fibrotic foci per linear field (Kenney, 1978). In seasonal anoestrus and during the transitional period before the onset of regular cyclicity, non-pathological nesting of glands should be distinguished from fibrosis.

Lymphatic lacunae. These lacunae are dilated lymphatic vessels occurring in

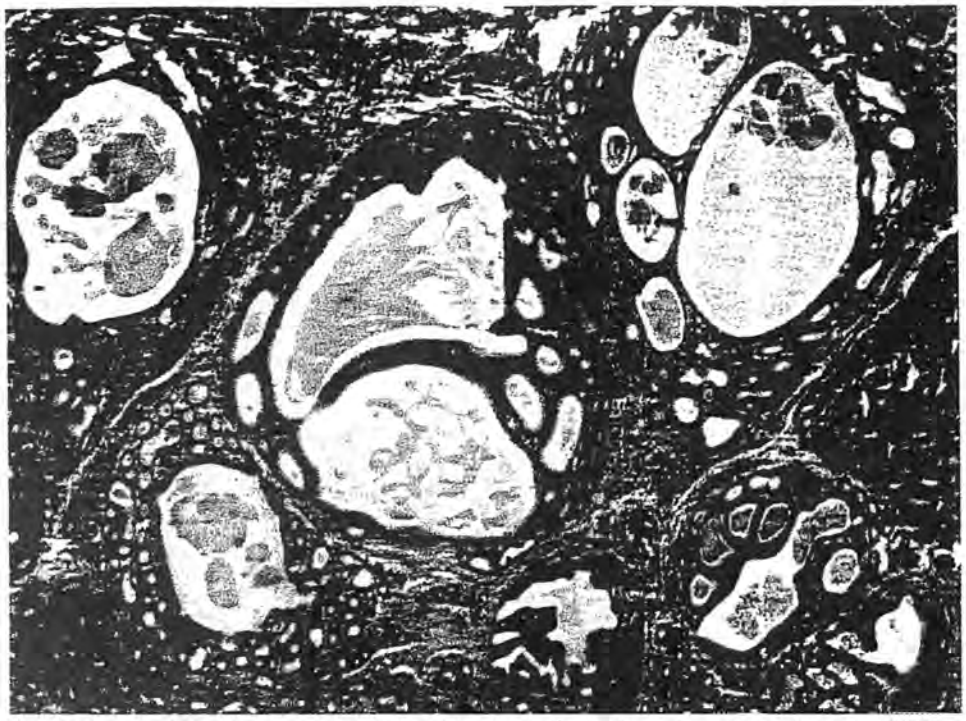

Fig. 5. Periglandular fibrosis and cystic glandular distension in the endometrium of a mare. HE. Low power.

the stratum spongiosum. They are found singly or in groups, and may be focal or diffuse. Individual lacunae may coalesce to form endometrial cysts which are visible by transrectal ultrasonography (Fig. 6) (Ginther, 1986). Lacunae must be distinguished from oedema induced by biopsy. It is likely that isolated cysts have little effect on pregnancy, but widespread changes are thought to reduce foaling rates (McKinnon *et al.*, 1987).

Cystic dilatation of glands. Cystic distension of glands commonly occurs in fibrotic nests (Fig. 5). Affected glands may be large enough to be detected ultrasonographically, but they are generally not as large as lymphatic endometrial cysts. During seasonal anoestrus, it is common to find widespread slight distension of glands containing inspissated secretion, but this usually disappears during the breeding season. Mares with widespread glandular distension in the absence of periglandular fibrosis tend to have decreased fertility.

Contagious Equine Metritis (CEM)

This disease, caused by *Taylorella equigenitalis*, a gram-negative microaerophilic coccobacillus, is characterized by a copious, watery mucopurulent discharge

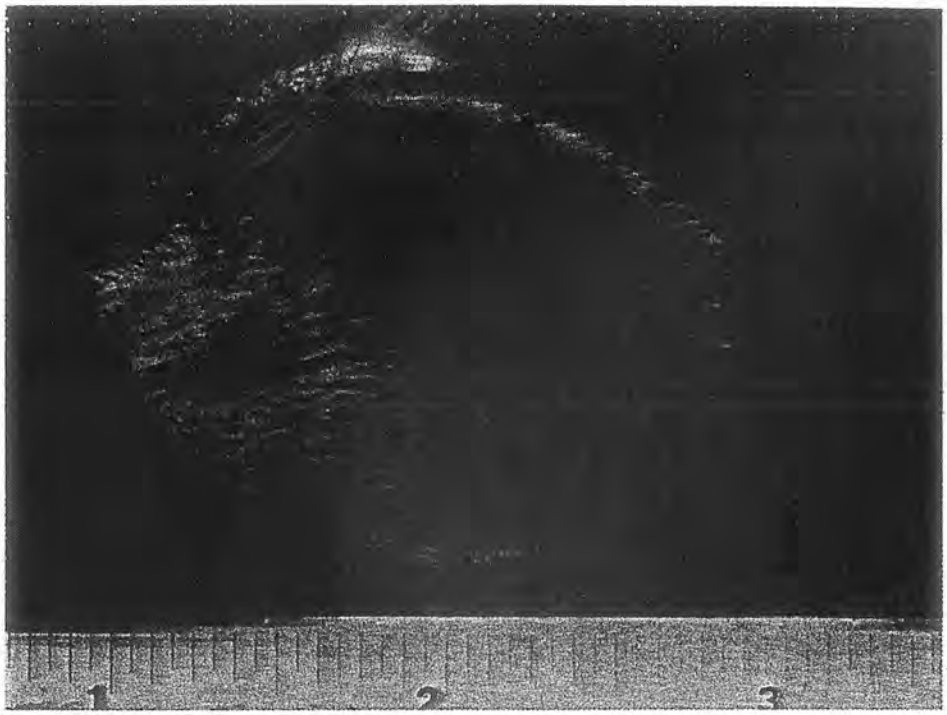

Fig. 6. Excised persistent hymen from a mare.

2 to 10 days after breeding to an infected stallion (Hughes, 1978). The disease persists for around 3 weeks. In some mares the only clinical sign is short oestrous cycles. The lesions in the tract are characteristic of acute endometritis (Acland and Kenney, 1983).

Endometrial Adhesions

Adhesions may be secondary to trauma resulting from dystocia or intrauterine infusion of caustic solutions, or they may be a sequel to severe endometritis. Adhesions may occur as single or multiple bands, partial or complete sheets, or tunnels between adjacent endometrial folds (Baker and Kenney, 1980). More extensive adhesions may interfere with fertility by causing pyometra or by restricting embryonic mobility within the uterus (McKinnon *et al.*, 1987).

Pyometra

Unlike pyometra in the cow, pyometra in the mare is not always accompanied by a retained corpus luteum and anoestrus. Affected mares may have regular cycles, or shortened cycles due to premature release of prostaglandin $F_{2\alpha}$. As much as 60 litres of exudate may accumulate in the uterus without producing signs of systemic disease or significant haematological changes (Hughes *et al.*,

1979). Histological examination of the endometrium reveals acute endometrial inflammatory changes. Endometrial damage is usually superficial with no involvement of the myometrium or perimetrium (Hughes *et al.*, 1979). In severely affected mares, the entire endometrium may be replaced by granulation tissue. In prolonged cases endometrial atrophy may be present, and in others fibrosis of the cervix may prevent drainage of the secretions.

Mucometra

This is a rare disease in the mare, but four cases have been reported in which cystic hyperplasia and lymphocytic infiltration of the endometrium were present (McEntee, 1990d).

Uterine Abscess

This is also rare but can result from dystocia, artificial insemination, severe metritis or uterine therapy. During the acute phase, clinical signs such as fever, neutrophilia and peritonitis may be present.

Uterine Haematoma

Haemorrhage may occur into the wall or lumen of the uterus or into the peritoneal cavity after trauma resulting from dystocia. Haemorrhage into the uterine lumen is not usually serious and resolves as the uterus involutes. However uterine artery rupture into the abdominal cavity results in death from exsanguination. Survival depends on whether the haemorrhage is contained within the broad ligament, resulting in haematoma formation. These peri-uterine haematomas regress with time, but the vessels frequently rupture in the same place during the next gestation with fatal results (Pascoe, 1979).

Uterine Neoplasia

Primary uterine neoplasia is rare in the mare. Solitary or multiple leiomyomas, 2·5–5 cm in diameter, which are often pedunculated, have been reported. Other tumours include lymphosarcoma (McEntee, 1990a), rhabdomyosarcoma (Torbeck *et al.*, 1980) and carcinoma (Gunson *et al.*, 1980).

Cervix

The cervix provides the final barrier against the entrance of microorganisms into the uterus. The cervix of the mare, unlike that of other domestic species, does not have transverse folds, but longitudinal folds are present, being continuous with the endometrial folds. A fold of mucosa extends to the floor of the vagina as a frenulum. The cervix of the mare may be digitally dilated at all stages of the oestrous cycle, for diagnostic or therapeutic purposes. The shape, size and consistency of the cervix varies markedly with the stage of the oestrous cycle and with pregnancy. During oestrus the cervix is short, moist,

oedematous, pink and relaxed and lies on the vaginal floor. By contrast, during dioestrus the cervix lengthens, is pale in colour and closed. This contracted condition is increased during pregnancy, when the cervix feels long and pencil-like and bulges into the anterior part of the vagina. During seasonal anoestrus the cervix may be closed or short, thin and open. Malfunctioning of the cervix will lead to uterine infection and infertility.

Cervicitis

Cervicitis is usually associated with vaginitis or endometritis. Apart from infection, it may result from contact with irritants such as air or urine in the vagina and uterine medications. Infrequently, pregnant mares develop cervicitis, giving rise to a vulvar discharge (Sertich, 1993). Local treatment may ensure the maintenance of pregnancy.

Cervical Laceration

Lacerations are thought to occur only at parturition, due to damage from foetal extremities, sometimes in the absence of dystocia. If severe, they may result in cervical incompetence, uterine contamination and endometritis.

Cervical Adhesions

These may result from trauma at parturition or from intrauterine infusion with irritant substances. The adhesions may be transluminal, resulting in excessive accumulation of fluid in the uterine lumen, or they may attach the cervix to the vaginal wall, preventing the cervix from closing effectively.

Developmental Anomalies

Cervical hypoplasia has been reported in a filly (Blanchard *et al.*, 1982), as has segmental aplasia of the paramesonephric duct resulting in absence of the cervix (Schlotthauer and Zollman, 1956). One case of a double cervix has been reported (Volkmann and Gilbert, 1989).

Vagina

A prominent transverse fold lies in the posterior part of the vagina, near the urethral orifice. In young mares this fold is more distinct, forming the hymen.

Pneumovagina

Aspirated air in the vagina is important and relatively common (Caslick, 1937), and can be treated surgically. It results primarily from poor vulvar and perineal conformation, in which the angle of the vulva is greater than 80° to the vertical and two-thirds of the vulvar lips lie below the level of the tuber ischii. Pneumovagina in thin mares may improve as the animal puts on weight.

Pneumovagina may lead to vaginitis, cervicitis and eventually endometritis, and in extreme cases air will be present in the uterus.

Urovagina

Accumulation of urine in the vagina may result from the same factors that cause pneumovagina. In some mares it only occurs at oestrus or after foaling, due to relaxation of ligaments. Injury to the urethral orifice or transverse fold at foaling may also lead to vesicovaginal reflux. An ectopic ureter may empty urine directly into any part of the reproductive tract and may not be detected at an early age. Cystitis or urolithiasis, which often leads to incomplete voiding or dribbling of urine, may cause urovagina. Urine pooling frequently leads to endometritis and, in the most severe cases, to pyometra (Pugh and Caudle, 1987).

Persistent Hymen

A complete hymen is more common in the mare than in other species (Fig. 6). It arises because the caudal section of the paramesonephric duct fails to fuse with the urogenital sinus. Thick secretions may accumulate cranial to the hymen and become infected.

Vulva

The vulva comprises two labia and a clitoris. The clitoris consists of a central part separated by grooves from two lateral parts. The glans, which is surrounded by a clitoral fossa, contains a median sinus and, in most (75%) animals, two shallow lateral sinuses. The sinuses and fossa harbour *Taylorella equigenitalis* in mares with CEM and therefore these sites are routinely swabbed in breeding mares (McAllister and Sack, 1990).

Neoplasia

Melanomas, often involving the anus, vulva and surrounding skin, are common in old grey horses (Fig. 7) (Levene, 1971). They are usually multiple, darkly pigmented and hairless, and range from 0·5–2 cm in diameter. All melanomas should be regarded as potentially malignant and may spread initially by the lymphatics. However, later involvement of the internal organs results from haematogenous spread (McFadyean, 1933).

Perineal Lacerations

Lacerations occur at parturition as a result of an oversized or malpositioned foetus, or of excessive manipulation at an assisted foaling. They are classified according to the degree of involvement of the surrounding structures, as follows. (a) First degree laceration: tear in the mucosa and skin of the vulva. (b) Second degree laceration: tear in the mucosa and submucosa of the dorsal

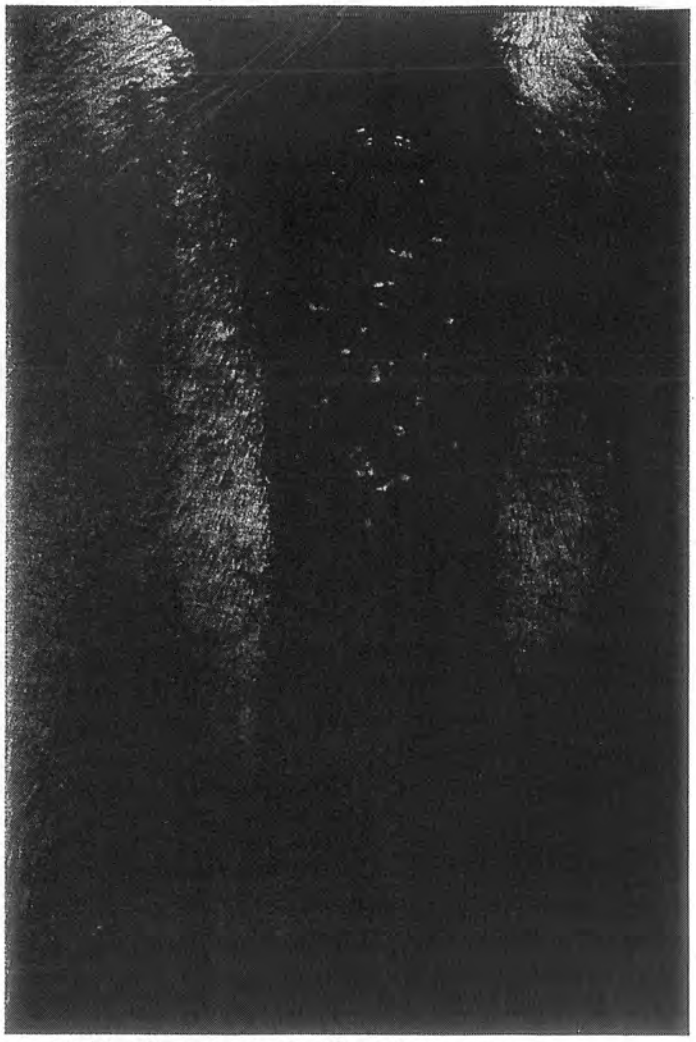

Fig. 7. Melanoma involving the anus and vulva of an old grey mare.

vestibule, the skin of the vulva, and part of the musculature of the perineal body. (c) Third degree laceration: tear through the vestibular wall into the rectum, disrupting the anal sphincter. (d) Perineal fistula: tear through the vestibular wall into the rectum.

Enlarged Clitoris

Male pseudohermaphrodites with testes and a 64XX karyotype frequently have an enlarged clitoris.

Coital Vesicular Exanthema

This infection, caused by equid herpesvirus-3, is venereally transmitted, resulting in the appearance of red papules and scabby erosions on the vestibule and vulva up to 10 days after coitus with an infected stallion. Within a further week, the lesions become pustular and may coalesce to form lesions 2 cm in diameter and 0·5 cm deep. Histological examination reveals intranuclear inclusion bodies in the epithelial cells surrounding the ulcers. The lesions heal within approximately 2 weeks, leaving depigmented areas. Occasionally the muzzle, nares and conjunctivae may be affected (Studdert, 1974).

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Special Article

Granulosa cell tumours in the mare: - a review of 9 cases

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Introduction

Granulosa cell tumours (GCT) are the most common ovarian neoplasm in the mare (Norris *et al.* 1968; Bosu *et al.* 1982; Meagher *et al.* 1977) and represent 2.5–4.4% of all equine neoplasms (Kerr and Alden 1974; Sundberg *et al.* 1977). These tumours are usually benign although malignant GCTs have been reported (Meagher *et al.* 1977; Gift *et al.* 1992). The average age of affected mares is 10.6 years (Meagher *et al.* 1977) but they have been reported in mares of all ages and reproductive states including neonates (Green *et al.* 1988), older foals (Hultgren *et al.* 1987) and pregnant and foaling mares (Stickle *et al.* 1975; Meagher *et al.* 1977).

These neoplasms often present clinically with behavioural changes and, on palpation of the genital tract, have unilateral ovarian enlargement, usually with atrophy of the contralateral ovary. Less commonly mares will present with lameness (Meagher *et al.* 1977), colic (Nyack and Johnson 1983) or weight loss (Nyack and Johnson 1983). The ovulation fossa of the enlarged ovary, which is normally palpable on the ventral aspect, is not present (Baker and Kenny 1980). Granulosa cell tumours tend to be secretory and the behavioural changes are caused by steroid secretion. Behaviour can be aggressive or stallion-like, constant or erratic oestrus, or anoestrus (Meagher *et al.* 1977; Perino and Didier 1985). Mares with stallion-like behaviour may also develop a crested neck, increased muscle mass and an enlarged clitoris (Clark 1975; Meagher *et al.* 1977). Approximately 50% of cases have elevated circulating concentrations of testosterone (>100 pg/ml) and 87% have elevated inhibin concentrations (McCue 1992). Stallion-like behaviour is associated with high circulating concentrations of testosterone, but the other types of behaviour are not obviously linked with reproductive hormone concentrations in blood (Stabenfeldt *et al.* 1979). Although oestradiol concentrations may be slightly elevated in mares with GCT, the high concentrations associated with the tumour in women (Besch *et al.* 1966) are rarely present. Measurement of oestradiol in mares with a GCT is of limited value. Atrophy of the contralateral ovary is thought to be caused by negative feedback from high circulating concentrations of inhibin or ovarian steroids (McCue 1992). However, occasional cases of cycling mares with a GCT have been reported (Hinrichs *et al.* 1990; McCue *et al.* 1991). Apart from these rare cases, plasma progesterone concentrations are almost invariably low (Stabenfeldt *et al.* 1979). It has been suggested that aromatisation of testosterone to oestradiol in mares with GCT may be reduced, as mares with very high testosterone concentrations do not necessarily have elevated oestradiol (Stabenfeldt *et al.* 1979). In the present study immunohistochemical techniques were used to detect cytochrome $P450_{C17}$, the steroidogenic enzyme responsible for synthesis of testosterone, and $P450_{arom}$, the enzyme which aromatises androgen to oestrogen, in neoplastic ovarian tissue from mares with GCTs. Transrectal ultrasonography has been reported not to be diagnostic for GCTs as they can appear multicystic (honeycombed), as a solid ovarian mass, or as a single large fluid filled cyst (White and Allen 1985; Hinrichs and Hunt 1990).

In the present study, therefore, both the transrectal ultrasonographic and gross pathological appearance of GCTs in 9 mares and ovarian haematomas in 4 mares, were recorded.

Case histories

Mares in the present study were aged 6–19 years and were

Fig 1: Echography of GCT Case 1, ***a) Two views of tumour showing small and larger cysts, b) Inactive contralateral ovary, c) Sectioned tumour.*** *Note pocket of sanguinous fluid attached to external capsule.*

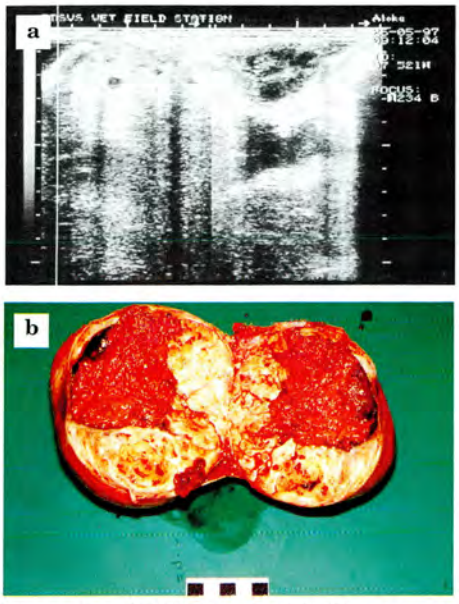

Fig 2: Echography of GCT Case 2, *a) Left screen shows solid tumour, right screen shows inactive contralateral ovary, b) Sectioned tumour with large area of inspissated blood and solid tissue containing very small cysts.*

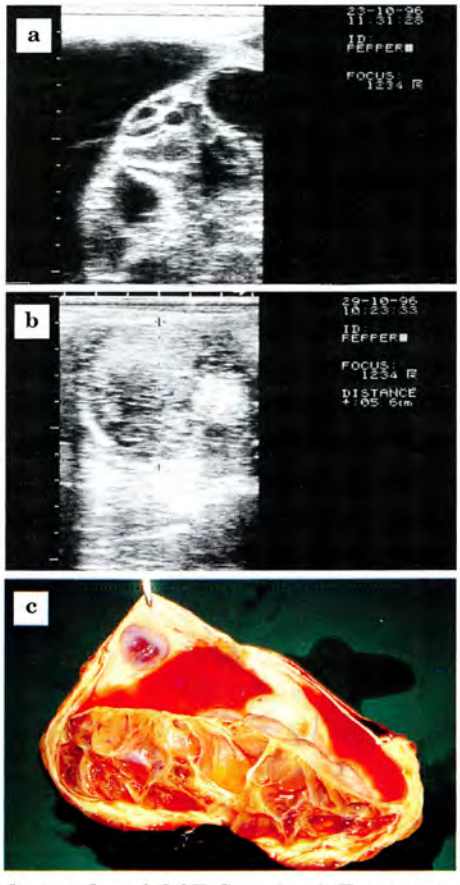

Fig 3: Echography of GCT Case 3, *a) Tumour containing large cysts, b) Large contralateral ovary containing 2 round echogenic structures, c) Sectioned tumour containing large irregular cysts.*

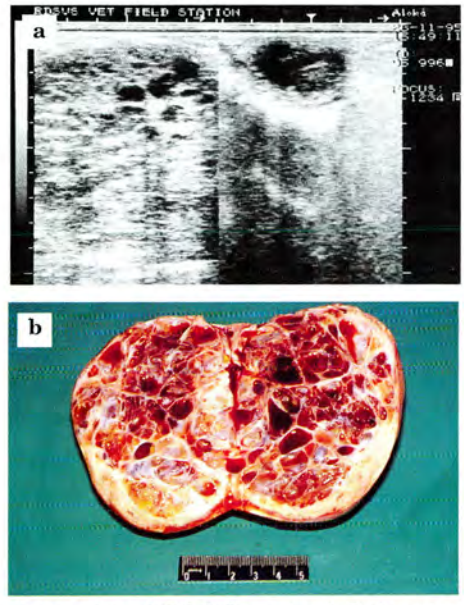

Fig 4: Echography of GCT Case 4, *a) Left screen shows tumour with small cysts and mottled heterogeneous appearance, right screen shows inactive contralateral ovary b) Sectioned tumour showing many irregular fluid-filled spaces with a few areas of dense stroma.*

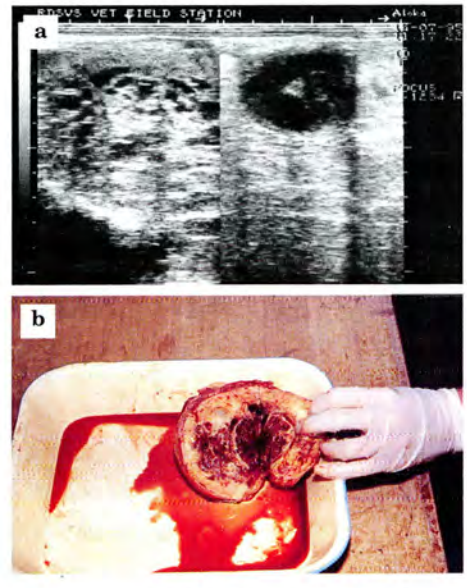

Fig 5: Echography of GCT Case 5, *a) Left screen shows tumour containing many small cysts and one large cyst, right screen shows inactive contralateral ovary, b) Sectioned tumour showing multiple cysts of variable size.*

either Thoroughbred, or of mixed breed. Five of the 9 mares with granulosa cell tumours displayed male-type behaviour, 3 were anoestrus and one was reportedly continuously in oestrus. All of the mares with stallion-like behaviour had elevated plasma concentrations of testosterone, as did one of the anoestrous mares. All of the other mares had basal concentrations of testosterone (Table 1).

TABLE 1: History and clinical findings of mares presented with an enlarged ovary

Mare	Age	Behaviour	Plasma testosterone (ng/ml)	Ovarian size (mm)	Weight (kg)	Contralateral ovary	Diagnosis
1	12	Stallion-like	>0.1	130 x 100	1.8	inactive	GCT
2	12	Stallion-like	>0.1	150 x 150	0.3	inactive	GCT
3	9	Stallion-like	>0.1	ND	ND	inactive	GCT
4	19	Anoestrous	<0.1	120 x 90	0.4	inactive	GCT
5	17	Continuous oestrus	<0.1	125 x 105	1.0	inactive	GCT
6	10	Stallion-like	>0.1	260	7.8	inactive	GCT
7	9	Anoestrous	<0.1	150 x 130	1.9	inactive	GCT
8	17	Anoestrous	>0.1	155 x 98	0.7	inactive	GCT
9	11	Stallion-like	>0.1	75 x 60	0.2	inactive	GCT
10	6	Normal cycles	ND	210 x 180 x 80	1.3	active	haematoma
11	11	Normal cycles	ND	145 x 115 x 103	1.5	active	haematoma
12	16	Normal cycles	ND	200	ND	active	haematoma
13	7	Normal cycles	ND	ND	ND	active	haematoma

ND = not determined.

Case 1

Echography revealed an enlarged affected ovary containing many small cysts with 2 large cystic pockets on one side of the ovary. In between areas of cysts there appeared to be dense mottled echogenic areas of fibrous tissue. The contralateral ovary was small and inactive (**Fig 1**). Pathology of the ovary revealed many small cysts and a large pocket of serosanguinous fluid attached to one side of the ovary.

Case 2

The affected ovary contained a very few small cysts <10 mm diameter (**Fig 2**). The rest of the tissue was uniformly echogenic. The contralateral ovary was small with follicles <5 mm. At surgery the abdomen contained approximately 15 litres serosanguinous fluid. Fibrin tags were present on the ovary and intestines. Cytology of the fluid revealed the presence of many macrophages and neutrophils. Gross examination revealed a necrotic soft area on the surface of the ovary with inspissated blood underlying it. The rest of the ovarian stroma was fibrous with small cysts.

Case 3

Echography revealed many large cysts in the ovary containing nonechogenic fluid (**Fig 3**), one of the cysts measuring more than 80 mm diameter. The contralateral ovary was large (56 mm) and contained 2 dense round echogenic structures. Because of the luteal-like appearance of this tissue plasma progesterone was measured but concentrations indicated that no luteal tissue was present. It is possible that these structures were resolved haematomas. Seven months later normal follicles were present in this ovary, next to the abnormal appearing tissue, and the mare was cycling normally. Grossly the neoplastic ovary contained irregular fluid filled spaces with serosanguinous fluid.

Case 4

Multiple small cysts were seen in the affected ovary on echography interspersed with large mottled areas of heterogeneous echogenicity (**Fig 4**). The contralateral ovary was small and inactive. Grossly, the affected ovary contained many irregular fluid filled spaces with a few dense areas of stroma.

Case 5

Many small cysts and a large cyst were apparent on echography of the affected ovary, separated by dense echogenic tissue (**Fig 5**). The contralateral ovary contained small follicles and appeared inactive. Examination of the ovary after removal revealed multiple cysts of variable size and much dense fibrous tissue.

Case 6

Echography showed many cysts, the most notable of which were approximately 10 mm (**Fig 6**). The rest of the tissue was of mixed echogenicity. The contralateral ovary was small and inactive. Pathology revealed many small to medium sized cysts.

Case 7

Two large cysts containing echogenic strands were seen on echography (**Fig 7**). The contralateral ovary was small and inactive. Pathology of the ovary revealed 2 large cysts surrounded by dense stroma containing small cysts.

Case 8

The affected ovary appeared multicystic on echography with one large cyst containing echogenic fluid (**Fig 8**). The contralateral ovary was small with multiple very small follicles. Gross examination showed the presence of multiple cysts with one large cyst containing serosanguinous fluid.

Case 9

The ovary appeared mottled with multiple small cysts and

one larger irregular hypoechoic area on echographical examination (**Fig 9**). The contralateral ovary was hypoplastic. Gross appearance was of a multicystic ovary with small cysts containing blood tinged fluid.

Case 10

Echography revealed a large ovary comprising one thick walled cyst filled with echogenic fluid (**Fig 10**). The contralateral ovary contained two 35 mm follicles. The mare had been referred because of an episode of colic 8 days previously. At surgery 7 litres sanguinous fluid was drained from the ovary. The removed ovary was hollow and thick walled. No normal ovarian tissue was present.

Case 11

This mare had a history of intermittent colic. The enlarged ovary contained echogenic material with no normal ovarian stroma visible (**Fig 11**). The contralateral ovary contained a corpus luteum. Gross examination showed that the ovary contained crumbly inspissated blood. No normal ovarian tissue was present.

Case 12

The affected ovary contained echogenic fluid with no normal ovarian tissue visible (**Fig 12**). The contralateral ovary contained a corpus luteum. The ovary was not removed and no pathology was therefore performed.

Case 13

The enlarged ovary was seen shortly after an ovulation. The mottled appearance showed echogenic fibrous clot with trapped anechoic areas of serum (**Fig 13**). The mare continued to cycle normally. The ovary was not removed and no pathology was therefore performed.

Immunohistochemistry

The steroidogenic enzymes $P450_{C17}$, responsible for converting progesterone to androgen, and $P450_{arom}$, responsible for converting androgen to oestrogen, were detected by use of specific antibodies as described previously (Watson and Thomson 1996; Rodger *et al.* 1998). High circulating testosterone was associated with distinct positive staining for $P450_{C17}$ (**Fig 14a**) whereas, mares with low testosterone had fewer cells which were positively stained (**Fig 14b**). Tumour tissue was associated with little staining for $P450_{arom}$. **Figure 14c** shows the presence of many granulosa cells which apparently lacked the presence of the enzyme.

Discussion

Differential diagnosis in mares with unilateral ovarian enlargement is most commonly between a granulosa cell tumour and an ovarian haematoma resulting from excessive bleeding at ovulation. Ovarian haematomas are normally smaller than those reported in the present study and regress over one to several months. They normally occur within the breeding season, the ovulatory fossa remains palpable and the mare continues to cycle normally. Ovaries containing small haematomas regain normal function as seen in **Case 13**. In **Cases 10** and **11** in the present study, both of which presented because of bouts of colic, no normal ovarian tissue remained and the ovary was replaced by an encapsulated sac of inspissated blood or with copious sanguinous fluid as in **Case 10**. None of these large haematomas had the typical echographic appearance of haematomas formed immediately after ovulation which present as diffusely echogenic mottled structures (Liu 1983) as seen in **Case 13**. Even these large haematomas resolve with time, as long as they do not get infected. The only indication for removal is if the mare is experiencing colic. It is probable that the dense echogenic areas in the normal ovary of **Case 3** were resolved haematomas.

In all of the mares with ovarian haematomas in the present report, the echographic appearance was easily distinguishable from the mares with GCTs. However, in some cases in which there has been less haemorrhage, it is reported that the appearance is indistinguishable from a GCT (Hinrichs and Hunt 1990). Two-thirds of the mares in the present series with GCTs had elevated testosterone in agreement with previous reports, where 50–90% of mares had elevated circulating concentrations of testosterone (Stabenfeldt *et al.* 1979; McCue 1992, respectively). In all but one of the mares in the present study high testosterone was associated with male-type behaviour as reported by Stabenfeldt *et al.* (1979). It is probable that proportionally more mares exhibiting stallion-like behaviour are presented by their owners for investigation than, for example, mares with GCTs which are in anoestrus. Cyst size varied markedly in the tumours from the classic honeycomb appearance to virtually solid or fluid-filled. There appeared to be no correlation between cyst size and hormone secretion and it was not therefore possible to predict whether plasma testosterone concentrations were elevated based on echographic appearance of the cysts. However, in testosterone-secreting tumours, there did appear to be greater expression of $P450_{C17}$, the enzyme responsible for conversion of progesterone to androgen than in tumours from mares with low circulating testosterone. Expression of aromatase in GCTs was uniformly low. This has been reported previously (Watson and Thomson 1996) and agrees with the conclusion reached by Stabenfeldt *et al.* (1979) that aromatisation of testosterone to oestrogen is low in equine GCTs.

Granulosa cell tumours can attain considerable size, the heaviest in the present study being almost 8 kg and one of 59 kg has been reported (Nyack and Johnson 1983). In accordance with other workers, the mares in the current series were aged mainly in the middle of the range 9–19 years. In all the mares of the current series with GCTs, the contralateral ovary was inactive. This has been the most common reported finding, although cases of GCTs in cycling mares have been reported (Hinrichs *et al.* 1990;

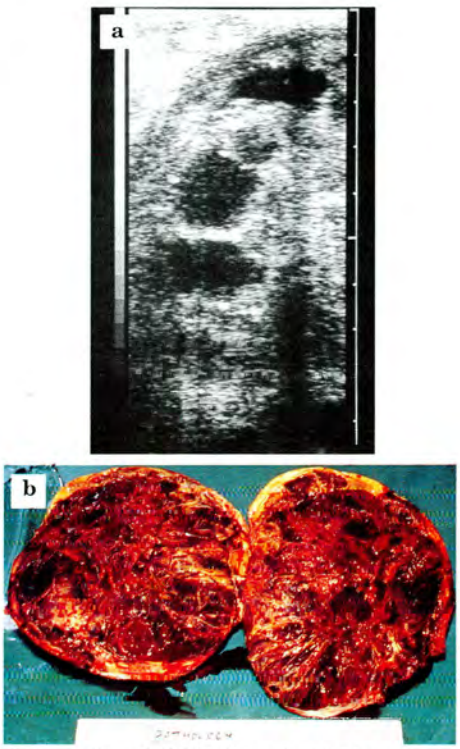

Fig 6: Echography of GCT Case 6, *a) Tumour containing many cysts b) Sectioned ovary containing many small to medium cysts.*

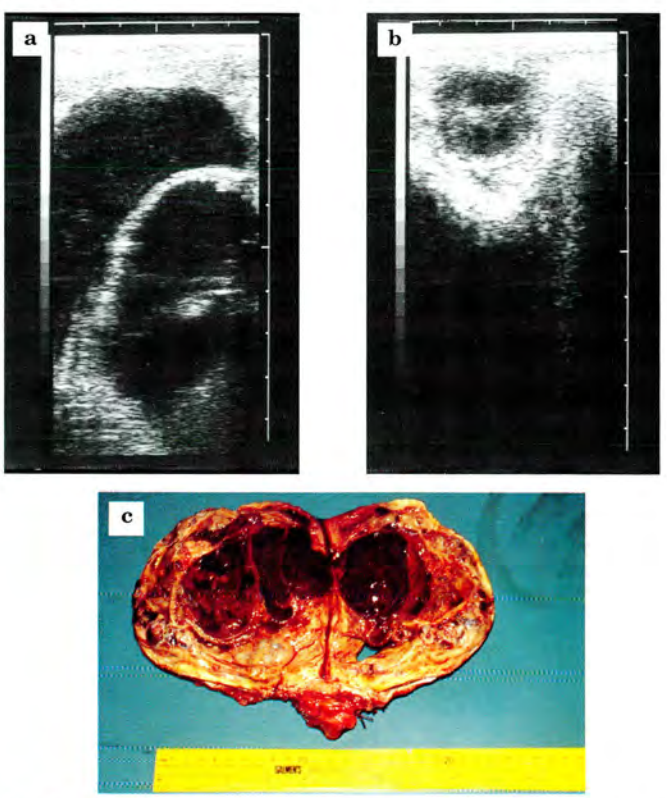

Fig 7: Echography of GCT Case 7, *a) Tumour comprising very large cysts, b) inactive contralateral ovary, c) sectioned ovary containing 2 large cysts surrounded by dense stroma with small cysts.*

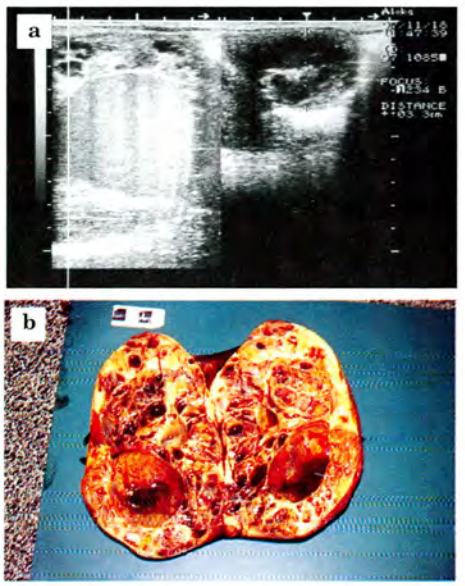

Fig 8: Echography of GCT Case 8, *a) Left screen shows small cysts surrounding a large central cyst filled with echogenic fluid, right screen shows the inactive contralateral ovary, b) Sectioned ovary with multiple cysts and one large central cyst containing sanguinous fluid.*

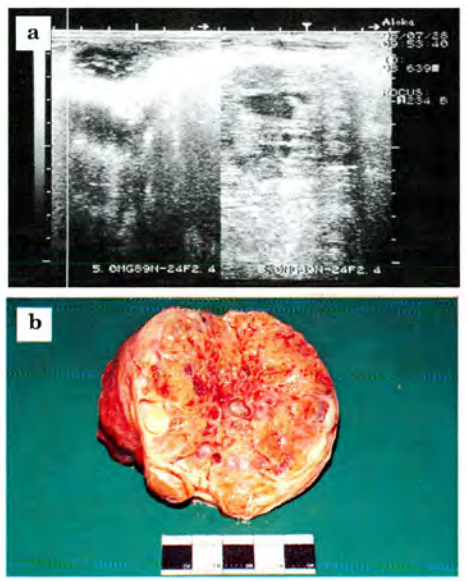

Fig 9: Echography of GCT Case 9, *a) Left screen shows inactive contralateral ovary, right screen shows tumour with mottled appearance containing small cysts and a central hypoechoic area, b) Sectioned tumour with small cysts.*

McCue *et al.* 1991). By contrast, all of the mares with ovarian haematomas in the present study were cycling and this can be a useful diagnostic finding in differentiating between these 2 conditions.

Another important differential diagnosis in mares, with one or both ovaries enlarged, is the multiple large follicles present on the ovaries during the transitional breeding season which fail to ovulate because of lack of gonadotrophic stimulation or of appropriate follicular

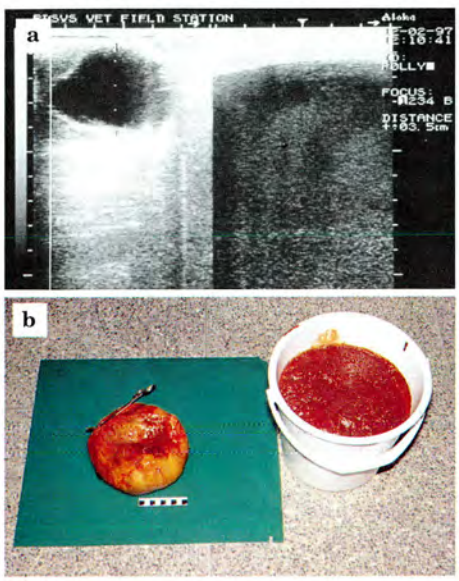

Fig 10: Echography of ovarian haematoma Case 10, *a) Left screen shows normal preovulatory follicle which is starting to 'point' towards the ovulation fossa, right screen shows a thick walled cyst containing echogenic fluid, b) Thick walled ovarian capsule showing drained sanguinous fluid in bucket.*

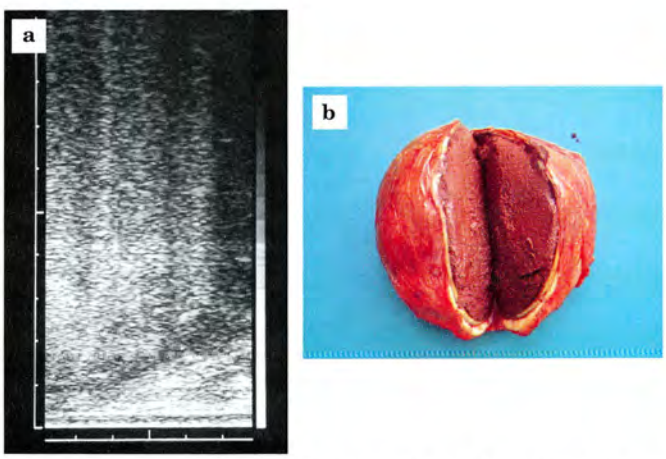

Fig 11: Echography of ovarian haematoma Case 11, *a) Ovary comprising solid echogenic material with no fluid movement on ballottement, b) Sectioned ovary comprising inspissated blood within the capsule.*

Fig 12: Echography of ovarian haematoma, Case 12. *Ovary contains echogenic fluid which swirled when balloted.*

Fig 13: Echography of ovarian haematoma Case 13. *Mottled appearance characteristic of echogenic fibrous clot with pockets of serum.*

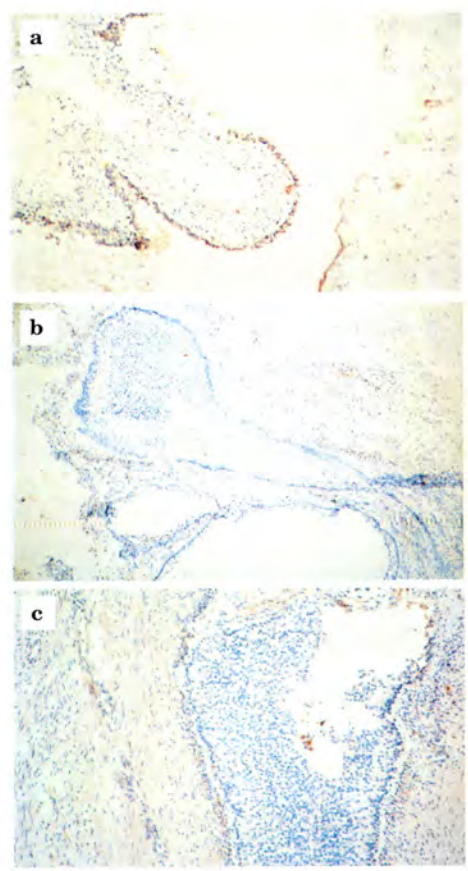

Fig 14: Immunostaining for steroidogenic enzymes. Positive staining is red a) Tumour secreting high concentrations of testosterone showing positive staining for P450c17 (x32) b) Tumour from mare with low circulating testosterone. Very few cells show positive staining (x20) c) Tumour showing little immunostaining for $P450_{arom}$ (x32). Note presence of many granulosa cells in the central part of the tissue which lack positive staining.

gonadotrophin receptors. These mares frequently show periods of prolonged oestrus until one of the follicles ovulates, after which normal cycling commences.

The mares in the present study were not monitored to determine the length of time to resumption of ovarian cyclical activity after tumour removal. However in most mares, ovarian activity is restored within 2–16 months after tumour removal (Pugh *et al.* 1985).

Acknowledgements

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Detection of high circulating concentrations of inhibin pro- and -αC immunoreactivity in mares with granulosa-theca cell tumours

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Keywords: horse; granulosa-theca cell tumour; inhibin; ovary; mare

Introduction

Granulosa-theca cell tumours (GTCT) are the most frequently occurring ovarian neoplasms in the mare (Meagher *et al.* 1977; Pugh *et al.* 1985). Although these can have certain features characteristic on transrectal ultrasonographic examination, it has been reported that there is no single typical appearance (Hinrichs and Hunt 1990; Watson 1999). Concentrations of plasma testosterone are elevated in around 50% of mares with GTCTs (Stabenfeldt *et al.* 1979; Watson 1999) and mares can show a variety of behaviours including anoestrus, constant or erratic oestrus, or stallion-like behaviour (Meagher *et al.* 1977; Watson 1999). The contralateral ovary tends to be small and inactive (Meagher *et al.* 1977; Hinrichs and Hunt 1990; Watson 1999) although not in all cases (Hinrichs *et al.* 1990).

Studies in women with granulosa cell tumours have established markedly elevated circulating levels of immunoreactive inhibin (Lappohn *et al.* 1989; Jobling *et al.* 1994; Boggess *et al.* 1997). Inhibins are dimeric proteins comprising an α-subunit and 1 of 2 β-subunits (β_A or β_B) forming 2 isoforms, inhibin A (α-β_A) and inhibin B (α-β_B). Most studies in normal cyclic or pregnant mares have reported circulating concentrations of immunoreactive inhibin (Bergfelt *et al.* 1991; Roser *et al.* 1994; Nambo *et al.* 1997). These earlier assays do not discriminate between the biologically active dimeric forms of inhibin and the free α-subunits, which may not be biologically active and which are present in the circulation in high concentrations (McNeilly *et al.* 1994).

A previous study reported elevated concentrations of immunoreactive inhibin in 87% of 39 mares with confirmed GTCTs (McCue 1992). However, concentrations in normal mares were not reported in this study for comparison. Use of a relatively nonselective assay may result in some normal mares being erroneously classed as affected because of extra-ovarian sources of the α-subunit. GTCT tissue has been shown to express mRNAs encoding inhibin α- and β_A-subunits (Piquette *et al.* 1990). A recent study by Christman *et al.* (1999) used an antibody raised against ovine inhibin A and reported concentrations higher than normal in 3 of 6 mares with GTCT.

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It has been reported that normally cycling mares have high circulating concentrations of a precursor form of the inhibin α subunit, measured as inhibin isoforms containing pro- and -αC immunoreactivity (Nagaoka *et al.* 1999). In women, measurement of inhibin isoforms with pro- and -αC immunoreactivity is a more reliable indicator of ovarian neoplasia than measurement of inhibin A (Burger *et al.* 1998), and leads to better discrimination between individuals in control groups and those with granulosa cell tumours. Immunohistochemical studies indicate that in the mare, the granulosa and theca cells of large follicles may secrete dimeric inhibin A, while granulosa cells of small follicles may secrete free α-subunit (Nagamine *et al.* 1998). Inhibin concentrations in the circulation vary with stage of oestrous cycle; therefore, any study of mares with GTCTs should include control groups of mares at various stages of the oestrous cycle. In the present study, we measured inhibin A and inhibin containing pro- and -αC immunoreactivity in plasma of mares with GTCTs and in mares at various stages of the oestrous cycle and in anoestrus in order to establish whether measurement of these hormones might be useful in diagnosis of GTCTs.

Materials and methods

Six of the mares referred into our clinic with suspected GTCTs were studied. The mares were Thoroughbred or Warmblood and weighed 500–550 kg. All of the mares had one large and one small ovary as determined by transrectal ultrasonography, and the large ovary contained cystic structures of various sizes. All of the mares were reported either to show aggressive, male-type behaviour or were unreliable when ridden. Blood was collected from the jugular vein into heparinised evacuated tubes. The tubes were centrifuged at 2000 *g* for 15 min and plasma was removed and stored at -20°C until analysis. After surgical removal of the large ovary, the clinical diagnosis of GCTC was confirmed by histological examination. Plasma was collected from another group of normally cycling mares of mixed breed weighing 350–520 kg on every second day during oestrus (n = 14), on the day of ovulation (n = 14), from 4 mares on Days 4, 7 and 10 after ovulation, and from 7 mares during winter anoestrus, prior to the appearance of the first 30 mm follicle on the ovaries.

The plasma samples were analysed for concentrations of

TABLE 1: Mean ± s.e. plasma concentrations of inhibin pro-αC (pg/ml) during the oestrous cycle, anoestrus, and in mares with granulosa cell tumours

	n	Inhibin pro-αC (pg/ml)
Granulosa cell tumour	6	3805 ± 1544[a]
Oestrus	14	238 ± 34.9[b]
Ovulation	14	326 ± 52.4[b]
Dioestrus	12	99 ± 11.4[c]
Anoestrus	7	85 ± 14.2[c]

Values with different letters are significantly different (P<0.005).

testosterone, inhibin A and inhibin isoforms containing pro- and -αC immunoreactivity. The testosterone assay was performed by a commercial laboratory (SCL Biosciences Ltd, Cambridge, UK). Concentrations of inhibin A and inhibin isoforms containing pro- and -αC immunoreactivity were measured using ELISAs as described previously (Groome *et al.* 1994, 1995) with some minor modifications (Riley *et al.* 2000). Standards used for inhibin A were a partially purified standard from human follicular fluid calibrated against recombinant inhibin A. For detection of inhibin pro- and -αC immunoreactivity, a highly immuno-purified preparation from human follicular fluid was utilised. The slopes of dilution curves with equine plasma were parallel to the standard curve. The intra- and interassay coefficients of variation were 7.6 and 9.8%, respectively for inhibin A, and 7.1 and 8.2%, respectively for inhibin pro-αC. The assay sensitivity for inhibin A was 15 pg/ml, and for inhibin pro- and -αC isoforms was 6 pg/ml.

Statistical analysis of the data

Mares with GTCTs and control mares at various stages of the cycle were compared by the Mann-Whitney test. Only the highest inhibin concentration during oestrus was included in the results. Findings were considered significant if P<0.05.

Results

Concentrations of inhibin isoforms containing pro- and -αC immunoreactivity were significantly higher (P<0.005) in mares with GTCT than in any of the other groups of mares (Table 1; Fig 1). All of the mares diagnosed as having GTCT were above the 95% reference interval (mean ± 2 s.d.) of the other groups except for the group sampled on the day of ovulation, when one of the mares with GCTC fell within the range of these normal mares.

Circulating inhibin A concentrations were very low or undetectable in the normal control mares but were high in 4 of the 6 mares with GTCTs (83, 81, 78 and 71 pg/ml). The 2 remaining mares had undetectable levels of inhibin A. Four of the 6 mares with GTCTs had elevated concentrations of testosterone in plasma (>0.1 ng/ml). The 2 GTCT mares with low/undetectable plasma concentrations of inhibin A were the same mares that had the low testosterone concentrations. These 2 mares also tended to have lower concentrations of inhibin isoforms containing pro- and -αC immunoreactivity than most of the other mares with GTCTs (Fig 1), but concentrations were still elevated compared with the control mares.

Cycle stage significantly influenced immunoreactive inhibin pro- and -αC subunit concentrations (Table 1). Concentrations

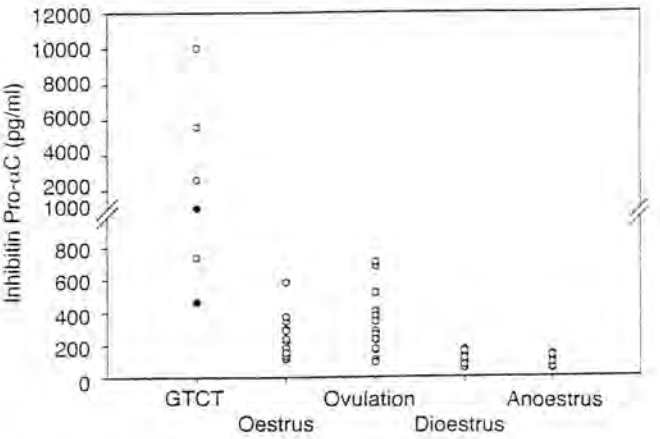

Fig 1: Concentrations of inhibin isoforms containing pro- and -αC immunoreactivity in mares with GTCT, and in mares in oestrus, on the day of ovulation, and in dioestrus and anoestrus. Closed circles represent the 2 mares with GTCT that had low concentrations of circulating testosterone.

were highest on the day of ovulation and were lower, but not significantly different, at their highest level measured during oestrus. Concentrations were significantly lower (P<0.005) during dioestrus and anoestrus.

Discussion

In the present study, we have shown, for the first time, that mares with GTCTs have high circulating concentrations of inhibin isoforms containing pro- and -αC immunoreactivity. Although there was some minor overlap with concentrations in mares in oestrus and, in particular, on the day of ovulation, concentrations during mid-dioestrus and anoestrus were significantly lower than the levels in mares with GTCTs. A previous study has shown that immunoreactive inhibin is elevated in 87% of mares with GTCTs (McCue 1992). However, there was no indication in that study of the range of concentrations in clinically-affected and normal mares, or whether false negatives were measured due to assay of α-subunits of extragonadal origin. It appears that inhibin isoforms containing pro- and -αC immunoreactivity is the predominant form of inhibin secreted by equine granulosa cells (Tanaka *et al.* 2000) and is present in high concentrations in the blood of normally cycling mares (Nagaoka *et al.* 1999). It is not surprising, therefore, that measurement of these isoforms appeared to provide a reliable way of detecting GTCTs in mares in the present study. A previous study has shown that both the granulosa cells and the polyhedral cells present in some equine GTCT produce inhibin α-subunits (Bailey *et al.* 2000). In association with an elevation in inhibin concentrations, plasma FSH concentrations are usually low in mares with GTCT (McCue 1992).

In our study, concentrations of plasma inhibin isoforms containing pro- and -αC immunoreactivity peaked on the day of ovulation as described previously (Nagaoka *et al.* 1999), presumably due to peritoneal absorption from the ruptured preovulatory follicle. Samples were collected only once on the day of ovulation in our study. However, inhibin pro- and -αC immunoreactivity peaks for 8–12 h after ovulation and then declines abruptly (Nagaoka *et al.* 1999); therefore, it is probable that peak

concentrations in some mares were missed with our sampling regimen. This would account for the lack of significant difference between peak oestrous concentrations and day of ovulation. The low concentrations measured in dioestrus and anoestrus support the findings of Nagamine *et al.* (1998) who showed that large follicles are the main source of dimeric inhibin in the mare. Furthermore, they were unable to detect inhibin α, β_A and β_B subunits in the equine corpus luteum by immunostaining. This indicates that the low concentrations of inhibin containing pro- and -αC immunoreactivity detected during dioestrus in the normal mares in our study probably originated from small follicles. The low levels measured in the mares during winter anoestrus reflects the absence of significant follicular activity in these animals.

We were unable to detect measurable circulating concentrations of inhibin A in the normally cycling mares. This is in agreement with Nagaoka *et al.* (1999) who used in effect the same ELISA. Nevertheless, this assay will detect inhibin A in follicular fluid, which is possibly due to the high concentrations at this site. However, high concentrations of inhibin A were measured in the plasma of 4 of the mares with GTCT. Similarly Christman *et al.* (1999), using a different assay, showed elevated concentrations of inhibin A in 3 of 6 mares with GTCT compared with control mares. Absolute values of inhibins are difficult to compare between studies due to the differences in the calibration of the inhibin standard preparations and the species used.

It is of note that concentrations of inhibin A were elevated in our study only in mares with high concentrations of plasma testosterone. This relationship has not been reported previously, but it is known that polyhedral-shaped cells are present in testosterone-producing tumours (Bailey *et al.* 2000). These cells are thought to be potential sources of androgen in mares (Nagamine *et al.* 1998), and were immunopositive for both α and β_A subunits which constitute inhibin A (Bailey *et al.* 2000). Therefore, these polyhedral-shaped cells may be the source of the high circulating inhibin A in the testosterone-producing GTCTs in the present study. Alternatively, the low inhibin A in mares with low testosterone may reflect an overall low level of hormone output by these tumours. Furthermore, the 2 mares with low testosterone also tended to have lower concentrations of inhibin isoforms containing pro- and -αC immunoreactivity, although levels were still elevated relative to controls. There was no relationship between tumour size and hormone output in our study and it is, therefore, unlikely that 2 different developmental stages of the tumours are represented. The higher level of reliability of measuring inhibin isoforms containing pro- and -αC immunoreactivity measurements compared with inhibin A in detecting the presence of a GTCT in a mare, is in agreement with results in women. Measurement of plasma concentrations of inhibin isoforms containing pro- and -αC have been shown to give better discrimination of affected from healthy women than inhibin A (Burger *et al.* 1998; Menon *et al.* 2000).

In conclusion, we have shown that measurement of circulating concentrations of inhibin isoforms containing pro- and -αC immunoreactivity in mares may be useful in confirmation of the presence of a GTCT. It appeared that the greatest chance of obtaining a false positive diagnosis was if a normal mare was sampled on the day of ovulation. However, ultrasonographic examination of the ovaries should determine whether a recent ovulation may have occurred. Inhibin pro-αC immunoreactivity may represent a novel diagnostic marker for this relatively common ovarian pathology in mares.

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THERIOGENOLOGY

EFFECT OF ASPIRATION OF FOLLICULAR FLUID ON SUBSEQUENT LUTEAL FUNCTION IN THE MARE

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ABSTRACT

Ultrasonography was performed daily on mares during estrus to assess follicular growth. All mares received 2,500 IU human chorionic gonadotropin (hCG) intravenously when the preovulatory follicle reached 35 mm in diameter. Follicular fluid was aspirated when a 35-mm follicle was detected (0 h) or 12, 24, or 36 h later. Blood samples were collected daily from the mares until they returned to estrus. When data were pooled for early (0 + 12 h) or late (24 + 36 h) aspirations, progesterone concentrations were lower on Days 2 ($P < 0.01$), 3 ($P < 0.01$), 4 ($P < 0.05$), and 5 ($P = 0.1$) in mares in which follicular fluid had been aspirated early. However, when progesterone concentrations were compared relative to administration of hCG, time of aspiration did not affect subsequent luteal function.

Key words: mare, aspiration, follicles, luteal function

INTRODUCTION

Embryo recovery rates are very low in older, subfertile mares (1). Even when Day-4 embryos from these mares are transferred to synchronized, reproductively normal recipient mares, embryo survival rates are low (2). It would be advantageous, therefore, to collect oocytes from these mares prior to ovulation and fertilize them in vivo or in vitro.

Recent advances in the technique of oocyte collection in mares permit oocytes to be collected from mares prior to ovulation, with an oocyte recovery rate of more than 70% (3,4). Successful heterogenous fertilization, in which an oocyte is fertilized in the recipient mare's oviduct, has been reported (4) and may now be applied in subfertile mares. To ensure that the donor's rather than the recipient's oocyte is fertilized, fluid is aspirated from the recipient's preovulatory follicle to remove the oocyte.

It is not known how the maturity of the follicle at the time of follicular fluid aspiration affects subsequent luteal function. Adequate luteal function is important in the donor, to ensure a normal return to estrus and thus allow further collection procedures, and in the recipient, because a normally functioning corpus luteum is necessary to achieve and maintain pregnancy. Aspiration of fluid from preovulatory follicles has been associated with subsequent luteal dysfunction in humans and

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primates (5, 6), and low concentrations of maternal luteal progesterone have been implicated in failures of in vitro fertilization and embryo transfer programs in humans (5). Recent work in mares has shown that plasma progesterone concentrations on Days 3 and 5 tended to be lower in mares for which fluid from the preovulatory follicle was aspirated 36 h after administration of hCG than in naturally ovulating mares (4). The present study investigated the effect on subsequent luteal function of aspirating fluid from preovulatory follicles before and at set times after intravenous administration of hCG to mares with a 35-mm preovulatory follicle.

MATERIALS AND METHODS

Twelve Standardbred, Thoroughbred, and Quarter Horse mares weighing 450 to 550 kg were used. The mares were teased daily with a stallion. Throughout estrus, the mares' ovaries were examined daily by ultrasonography per rectum. All mares received 2,500 IU i.v. hCG on the day a 35-mm follicle was detected.

The mares were assigned randomly into one of three groups of four mares each. Aspirations of follicular fluid were performed at 0 (for mares aspirated at 0 h, hCG was administered 30 min after aspiration), 12, and 24 h after detection of a 35-mm follicle. At a subsequent cycle, follicular fluid was aspirated from three of the mares 36 h after detection of a 35-mm follicle and administration of hCG.

Blood samples (10 ml) were also collected daily from the jugular vein into evacuated heparinized tubes until the mares returned to estrus. The blood was centrifuged at 2000 g for 10 min and the plasma was stored at -20°C until assayed for progesterone.

Blood samples were collected daily from five of the mares from the time of a subsequent ovulation at which follicular fluid was not aspirated until they returned to estrus.

Procedure for Aspiration of Follicular Fluid

A previously described colpotomy procedure was employed (3). Mares were sedated with xylazine, and butorphanol was administered as an analgesic agent. After cleansing of the perineum and surgical preparation of the flank, an incision was made in the anterior vagina and the ovary located and fixed by inserting the hand into the peritoneal cavity. After administration of local anesthesia, a stab incision was made in the flank and an 18-gauge, 18-cm spinal needle was inserted in the peritoneal cavity. The stilette was removed, the needle was inserted into the follicle. Fluid was drawn from the follicle by suction applied using a 30-cc syringe attached to an extension tube.

Ultrasonography was performed the day after follicular fluid was aspirated to confirm the presence of a corpus hemorrhagicum.

Plasma Progesterone Assay

Plasma was assayed for progesterone using a Coat-a-Count progesterone kit.[a] Standards were suspended in plasma from an ovariectomized mare and

[a] Diagnostic Products Corporation, Los Angeles, CA.

progesterone concentrations were assayed in unextracted plasma using a solid-phase radioimmunoassay. The main cross-reactivities of the antibody were with 11-deoxycorticosterone (1.7%), 11-deoxycortisol (2.4%), 20 alpha-dihydroprogesterone (2%), 5 beta-pregnan-3,20-dione (1.3%). Cross-reactivities with other steroids tested were less than 1%. The sensitivity of this assay was 25 pg/tube. Intra- and inter-assay coefficients of variation were 6 and 9%, respectively.

Statistical Analysis

A one-way analysis of variance was employed to evaluate the effect of time of aspiration of follicular fluid or administration of hCG on progesterone concentrations. Where appropriate, least significant difference of means were compared.

RESULTS

One mare which had been aspirated at 0 h failed to develop a corpus hemorrhagicum and maintained basal plasma concentrations of progesterone. One mare, aspirated after 24 h, had a second ovulation on Day 4. These two mares were excluded from further analysis. The data were analyzed in two ways: progesterone concentrations were compared 1) when day of aspiration of follicular fluid was designated Day 0 or 2) when day of administration of hCG was designated as Day 0.

Ultrasonography revealed a normal corpus hemorrhagicum on the ovaries of all other mares by 24 h after aspiration of follicular fluid using the criteria of Pierson and Ginther (7).

Figure 1 shows plasma progesterone concentrations in mares aspirated 0, 12, 24 or 36 h after detection of a 35-mm follicle. Day 0 was designated as day of aspiration. Progesterone concentrations in samples obtained on Day 2 were lower ($P < 0.001$) in mares aspirated at 0 h than in the other groups. Concentrations in samples from mares aspirated at 12 h were also significantly lower ($P < 0.05$) than for mares aspirated at 24 h. On Day 3, samples from mares whose follicular fluid was aspirated at 0 h tended to have lower progesterone concentrations than samples from mares aspirated at 24 h ($P < 0.07$). Also, samples from mares aspirated at 12 h had lower concentrations ($P < 0.05$) than samples from mares aspirated at 24 h. On Day 4, samples from mares aspirated at 0 h had lower ($P < 0.05$) progesterone concentrations than samples from mares aspirated at 24 h and samples from mares aspirated at 12 h had lower ($P < 0.06$) progesterone concentrations than samples from mares aspirated at 24 h. Progesterone concentrations in mares aspirated at 36 h tended to be higher than mares aspirated at 0 and 12 h on Days 3, 4 and 5, but were not significantly different. After Day 5, there were no significant differences in plasma progesterone concentrations among groups.

When the data from early (0 h and 12 h) and late (24 h and 36 h) preovulatory follicles were pooled, progesterone concentrations were significantly lower on Day 2 ($P < 0.01$), Day 3 ($P < 0.01$), and Day 4 ($P < 0.05$), and tended to be lower on Day 5 ($P = 0.1$) for samples from mares in which fluid from early preovulatory follicles was aspirated as compared with samples from mares in which fluid from late preovulatory follicles was aspirated.

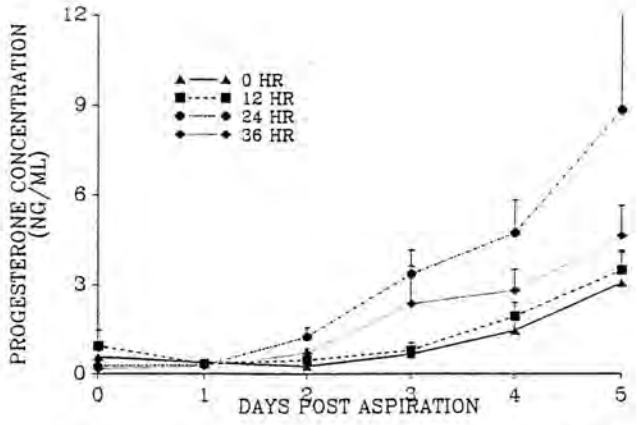

Figure 1. Concentrations ($\bar{x} \pm$ SEM) of plasma progesterone after aspiration of follicular fluid from mares 0, 12, 24 and 36 hours after detection of a 35-mm follicle.

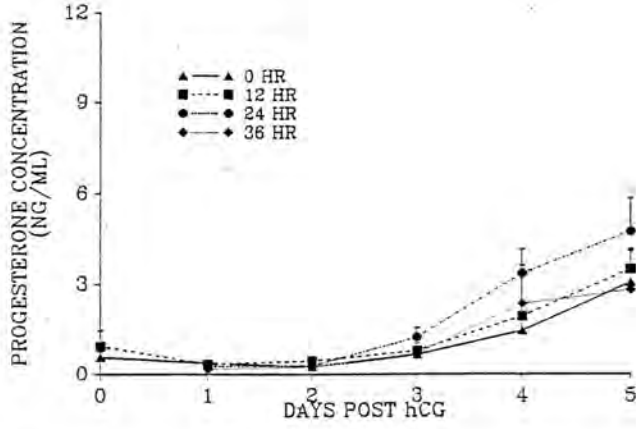

Figure 2. Concentrations ($\bar{x} \pm$ SEM) of plasma progesterone after administration of hCG to mares. Follicular fluid was aspirated 0, 12, 24 or 36 hours after administration of hCG.

Length of luteal phase (day of aspiration to return to estrus) was not affected by aspiration of follicular fluid (range 16 to 20 d). When day of administration of hCG was designated as Day 0, timing of aspiration of follicular fluid did not significantly affect subsequent progesterone concentrations (Figure 2). Progesterone concentrations in blood samples collected from five mares after ovulation were compared with all mares which underwent aspiration of follicular

fluid. If day of ovulation was assumed to occur on Day 2 after hCG treatment in aspirated mares, then concentrations of progesterone during the luteal phase were not significantly different in these two groups of mares (data not shown).

DISCUSSION

Administration of hCG (2,500 IU) in mares when the preovulatory follicle reaches 35 mm induces ovulation between 24 and 48 h later (8). Thus, in our study, the follicles from which fluid was aspirated 24 or 36 h after administration of hCG were very close to ovulation. It is known that characteristic changes occur within horse follicles as they approach ovulation. Numbers of luteinizing hormone (LH) receptors decrease (9), accompanied by increases in concentrations of progesterone, testosterone and prostaglandins in the follicular fluid, whereas concentrations of estradiol tend to decrease (10,11). In the cow, similar patterns have been observed in steroid concentrations in preovulatory follicles (12), and it is thought that the effect of exposure to elevated preovulatory concentrations of plasma LH results in inhibition of the aromatizing capacity of the granulosa cells. This causes depletion of substrate for estrogen synthesis (12-14), while the granulosa cells differentiate into lutein cells. In our study, because fluid from the preovulatory (0 h) horse follicles was aspirated before treatment with hCG, it is probable that the granulosa cells were still capable of aromatization and had not yet fully developed their capacity for progesterone production. McNatty (15) found that at least two criteria must be satisfied before follicles can form fully functional corpora lutea. There must be adequate numbers of granulosa cells and there must be sufficient LH receptors. From the work of Fay and Douglas (9), it seems likely that adequate numbers of LH receptors were present on the 35-mm follicles. Perhaps the numbers of granulosa cells present in the follicles from which fluid was aspirated at 0 and 12 h were not sufficient to produce adequate concentrations of progesterone during early diestrus. When groups were compared relative to time of administration of hCG, no differences in progesterone concentrations were discerned.

McKinnon et al. (4) reported that progesterone concentrations in samples drawn on Days 3 and 5 tend to be lower for mares after aspiration of fluid from follicles 36 h after administration of hCG than for mares with normal ovulations. However, the follicles were flushed with large volumes of buffered saline to facilitate oocyte recovery, and it is likely that some granulosa cells were removed by this technique. In our study, there were no differences in plasma progesterone concentrations between mares that underwent aspiration of follicular fluid and mares that underwent normal ovulation.

Results of our study indicate that luteinization and production of progesterone relate to time of administration of hCG rather than to time of aspiration of follicular fluid. If so, follicular fluid could be aspirated at any time after administration of hCG to a mare with a 35 mm follicle without affecting subsequent luteal function.

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ELSEVIER

CONTROL OF FOLLICULAR DEVELOPMENT AND LUTEAL FUNCTION IN THE MARE: EFFECTS OF A GnRH ANTAGONIST

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ABSTRACT

Control of the equine estrous cycle was studied by suppressing gonadotropin secretion by administration of a GnRH antagonist to cyclic pony mares. Four mares received vehicle (control cycle) or a GnRH antagonist, Antarelix™ (100 μg/kg) on Day 8 of diestrus, and blood samples were collected at 15-min intervals from 0 to 16 h, 24 to 36 h, and daily until the next ovulation. Ovarian activity was monitored by transrectal ultrasonography, and measurement of plasma concentrations of progesterone and estradiol. Antagonist treatment eliminated large diestrous pulses of LH. Progesterone concentrations had fallen significantly in all mares by the day after treatment and, in three of the four mares, remained low until luteolysis. However timing of luteolysis (ie., progesterone concentrations <1 ng/mL) was not affected by antagonist treatment. The preovulatory surges of estradiol and LH were significantly delayed in the treatment cycle, as was the appearance of a preovulatory follicle >30 mm. Cycle length was significantly longer during the treatment than the control cycle. These results show that treatment of diestrous mares with a GnRH antagonist attenuated progesterone secretion, indicating a role for LH in control of CL function in the mare, and delayed ovulation presumably because of lack of gonadotropic support.

Key words: mare, GnRH antagonist, follicle, corpus luteum

INTRODUCTION

The luteotropic control of the corpus luteum (CL) varies between species. In women, and old and new world primates, GnRH antagonist treatment has demonstrated that luteinizing

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hormone (LH) drives CL function (9, 13, 18, 39). In ruminants, support of the CL may be more complex, but in general LH is regarded as the primary luteotropin (28). In the mare the control of the CL remains to be elucidated. There is some evidence that LH is luteotropic, because administration of hCG and GnRH to diestrous mares results in increases in plasma progesterone concentrations (22, 23, 38). Also the CL of mares treated with an antiserum against an equine pituitary extract were lighter in weight than those of control mares (32) and appeared, macroscopically, to be regressing (33). However, although it is known that equine luteal cells bind LH (3), no close temporal relationship has been found between pulses of LH and progesterone in peripheral blood of pregnant and nonpregnant mares (31, 38). In vitro studies using short-term incubations of luteal cells have demonstrated either an increase (23) or no change (3, 7) in secretion of progesterone after addition of LH, but it has been shown that results obtained by in vitro culture are affected by the methods used for cell dissociation (3).

GnRH antagonist treatment allows immediate specific suppression of LH and FSH release in vivo and has been used to study pituitary-ovarian relationships in a number of species. No such studies have been described in the mare although the use of a GnRH antagonist has been reported to suppress ovarian activity and synchronize ovulation (30). A close correlation has been demonstrated between hypothalamic GnRH release and pituitary LH and FSH secretion in luteal-phase mares (20), and LH secretion is reduced by 90% to 100% in ovariectomized mares actively immunized against GnRH, showing that LH release in the mare is highly dependent on stimulation by GnRH (15). By contrast FSH is reduced by only 50% in GnRH-immunized mares (15), and therefore it has been suggested that FSH secretion is controlled only in part by GnRH.

In the present study, mares were treated with a potent GnRH antagonist in mid-diestrus at a time when progesterone output by the CL is maximal. This approach to studying the action of LH in vivo in the mare by suppressing its release is complicated by the long half-life of LH in this species (19), which means that plasma concentrations will not drop quickly after antagonist treatment. However, we presumed that the pulsatile release of LH would be eliminated after treatment, which, together with the slow decline in LH, should give an indication of the role of LH in CL support in the mare. Furthermore it was predicted that LH, and possibly FSH, concentrations would be low by the time that follicles should be growing for the mare's next estrus, and so treatment with the antagonist would also give information on the requirement of gonadotropins for folliculogenesis.

MATERIALS AND METHODS

The study was carried out between May and September. In the first instance, three ovariectomized mares (225 to 450 kg) were treated with the GnRH antagonist to determine the effectiveness of the treatment in suppressing gonadotropin release. Blood samples (10 mL) were collected into heparinized evacuated tubes once daily for 7 days before antagonist treatment. On the day of treatment the mare received a subcutaneous injection of the potent water-soluble GnRH antagonist, Antarelix™ ([N-Ac-D-Nal(2)^{1}, D-pCl-Phe^{2}, D-Pal(3)^{3}, D-(HCi)^{6}, Lys(iPr)^{8}, D-Ala^{10}] GnRH; 100 μg/kg in 10 mL 5% mannitol, Europeptides, Argenteuil, France). In one mare, daily blood sampling was continued for 7 days, and in the other two mares samples were collected for 32 days after antagonist treatment.

To study the effect of the antagonist on intact mares, four cyclic pony mares aged 7 to 18 years and weighing 198 to 298 kg were used. The ovaries of the mares were examined daily by transrectal ultrasonography during estrus until the day of ovulation (Day 0). The experiment was carried out during two cycles. In the first cycle, on Day 8 after ovulation, the mares received a subcutaneous injection of vehicle (10 mL 5% mannitol). In the second cycle the same mares were given a subcutaneous injection of the GnRH antagonist (100 μg/kg) on Day 8 after ovulation. Blood samples (20 mL) were collected via indwelling jugular cannulae into evacuated heparinized tubes at 15-min intervals for 16 h (Day 8) and also between 24 and 36 h (Day 9) after injection of either the drug or the vehicle. Blood samples were then collected daily by venipuncture until they returned to estrus and ovulated. The diameter of the CL and follicular activity were monitored by transrectal ultrasonography on every second day throughout the cycle until estrus, when the mares were scanned daily.

Blood samples were kept on ice until being centrifuged at 2000 x g for 15 min at 4°C. Plasma was decanted and stored at -20°C until assay. Daily samples and samples collected every hour during the sampling periods on Days 8 and 9 were assayed for progesterone. Daily samples were assayed for estradiol-17β and FSH, and LH was measured in all samples including the samples collected at 15-min intervals.

Radioimmunoassays

With the exception of estradiol, concentrations of hormones were measured by radioimmunoassay directly in plasma without extraction using techniques previously validated for LH (38), progesterone (8, 24) and FSH (34). For estradiol-17β, samples were extracted once in diethyl ether and assayed as previously described (17). The mean extraction efficiency for estradiol was 88% ± 1.1% (SEM.). In the FSH assay, a different rabbit anti-human FSH antibody (NIDDK-anti-hFSH-6) to that reported previously was used, at a final dilution of 1:10,000. The main cross-reactivity of this antibody was the hTSH (1%). The main cross-reactivities of the progesterone antiserum were with 5-pregnan-3,20 dione (9.5%), 11-deoxycorticosterone (6.2%) and 17-hydroxyprogesterone (3.4%). Progesterone standards were prepared in ovariectomized mare plasma. Assay sensitivities were 0.6 ng/mL for LH, 0.5 ng/mL for progesterone, 8 pg/mL for estradiol and 0.5 ng/mL for FSH, with intra- and interassay coefficients of variation of 13.8% and 14% for LH, 9.0% and 12.6% for progesterone, 4.6% and 7.8% for estradiol, and 6.3% and 10.2% for FSH, respectively. For each hormone, displacement curves produced by serial dilutions of plasma and spiking of samples containing low concentrations of the respective hormone were parallel to the standard curves. The average recovery of a known amount of hormone added to equine plasma was 92% for LH, 100% for progesterone, 91% for estradiol and 94% for FSH.

Statistical Analyses

Differences in cycle length, CL diameter until luteolysis, progesterone concentrations in hourly samples collected on Days 8 and 9, and mean LH concentrations in the serial blood collections were compared using the Kruskal Wallis Analysis of Variance. Luteolysis was defined as starting when progesterone declined to concentrations below 1 ng/mL. Daily progesterone concentrations in control and treatment cycles were compared using the Wilcoxon

signed rank test. Pulses of LH were identified as previously described (38). Differences in time to a preovulatory rise in gonadotropin and estradiol concentrations were compared by the Kruskal Wallis Analysis of Variance. The start of the preovulatory rise was defined as the day when concentrations first exceeded mean +1 SEM of daily concentrations for each mare, and when the increase was maintained for at least 3 days.

RESULTS

In the ovariectomized pony mares, mean concentrations of FSH and LH had fallen to approximately 40% and 10% of pretreatment levels, respectively, by Day 3 or 4 after antagonist treatment and remained at this level for around 7 days. By 32 days after antagonist treatment, concentrations of LH were still only around 50% of those collected before treatment, but FSH had returned to pretreatment concentrations.

Estrous cycles of the intact mares were longer during the treatment than the control cycle (33.5 $\pm$ 3.8 days vs. 24.0 $\pm$ 1.1 days; P = 0.01). The major factor in lengthening of the treatment cycle was caused by extension of the follicular phase (time between luteolysis and ovulation 17.5 $\pm$ 3.1 days vs. 6.0 $\pm$ 0.9 days; P<0.01) rather than by premature luteolysis. The prolongation of time to ovulation was due to a delay (P<0.05) in appearance of a large preovulatory follicle (>30 mm) on the ovaries during the treatment compared with the control cycle (Day 27.5 $\pm$ 4.2 vs. 18.8 $\pm$ 0.9; Figure 1), rather than the actual time taken for this follicle to grow and ovulate as determined by ultrasonography (5.8 $\pm$ 1.1 days vs. 5.5 $\pm$ 0.7 days). The diameter of the CL tended to be less between treatment (Day 8) and luteolysis (progesterone <1 ng/mL) in the treatment cycle (21.3 $\pm$ 2.6 mm) than during the control cycle (27.0 $\pm$ 2.5 mm), but this failed to reach significance (P = 0.16).

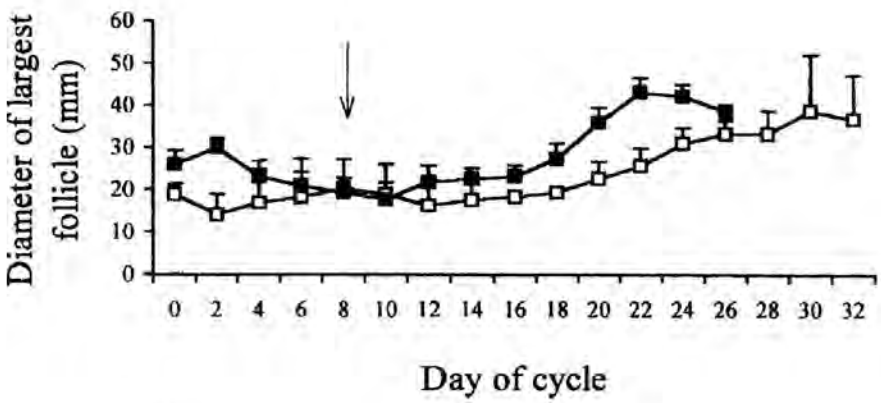

Figure 1. Mean ($\pm$ SEM) diameter of the largest ovarian follicle in 4 mares during a control (solid squares) and treatment (open squares) cycle. Arrow shows day of GnRH antagonist treatment.

In three of the mares, daily progesterone concentrations were significantly lower ($P<0.01$) after antagonist treatment compared to the control cycle (Figure 2). The decrease in progesterone concentrations was significant in the mean of the hourly samples of all 4 mares collected on the day after antagonist treatment (11.4 ± 2.5 ng/mL vs. 7.6 ± 2.2 ng/mL; $P = 0.01$) compared with the control cycle (11.2 ± 1.6 ng/mL vs. 10.6 ± 1.1 ng/mL). In the mare in which a decrease in progesterone concentrations was not maintained after antagonist treatment, a 40-mm follicle that had fibrin tags in the antrum and a thick echogenic wall was recorded from Day 5 of the treatment cycle. This follicle persisted throughout the cycle, regressing by Day 16. The progesterone concentrations in this mare were considerably higher than those in the other mares, and, although there was a decline in progesterone concentrations on Day 9 comparable to other mares, the levels rose again by Day 10 and remained high (16 to 23 ng/mL) until Day 15. Concentrations fell to below 1 ng/mL on Day 18. Results from this mare were excluded from the analysis of mean daily progesterone samples after treatment.

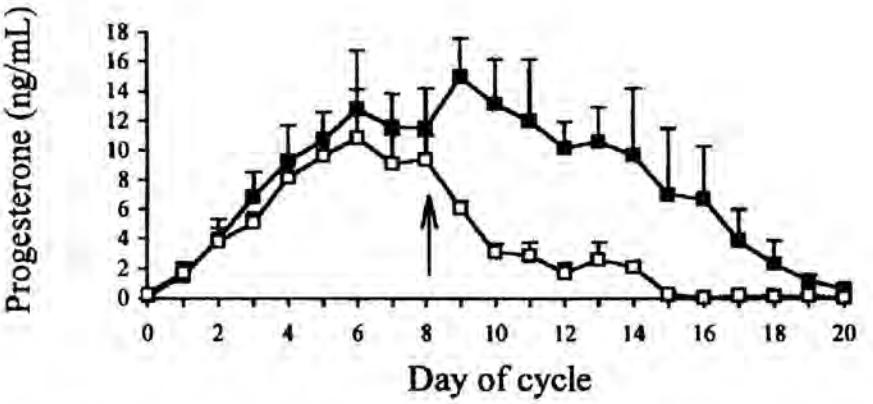

Figure 2. Mean (± SEM) plasma progesterone concentrations in 3 mares (excludes a mare with an anovulatory luteinized follicle) for 20 days after ovulation in the control (solid square) and GnRH antagonist treatment (open square) cycle. Arrow shows day of treatment.

Concentrations of FSH in daily samples after antagonist treatment were not significantly different in treatment and control cycles, but the start of the diestrous peak tended to be later ($P=0.07$) in treatment (Day 14.5 ± 1.0) than in control (Day 10.8 ± 1.3) cycles (Figure 3). Daily LH concentrations were very variable among mares and cycles. Mean concentrations after antagonist treatment were not significantly lower than during the control cycle.

Concentrations of LH started rising for the preovulatory surge at a significantly ($P<0.01$) later time during the treatment cycle (Day 25.5 ± 1.2) than during the control cycle (Day 19.8 ± 0.8; Figure 4). In the serial samples, mean LH was similar in both groups on Day 8 (2.4 ± 0.3 ng/mL and 2.2 ± 0.3 ng/mL in the control and treatment cycles, respectively), but tended to be

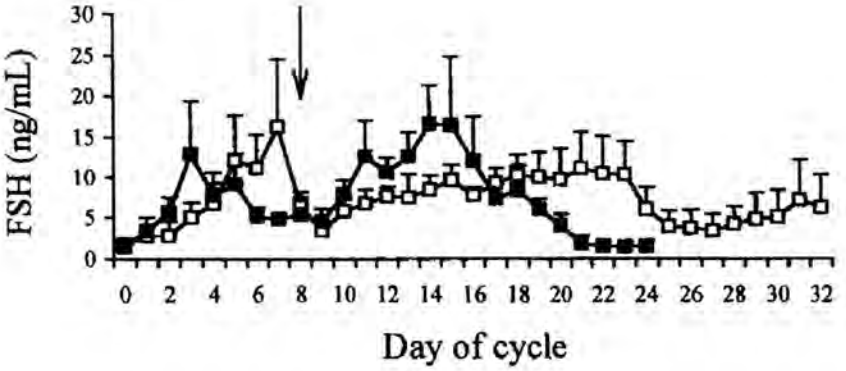

Figure 3. Mean (± SEM) concentrations of FSH in 4 mares during a control (solid squares) and GnRH antagonist treatment (open squares) cycle. Arrow shows day of treatment.

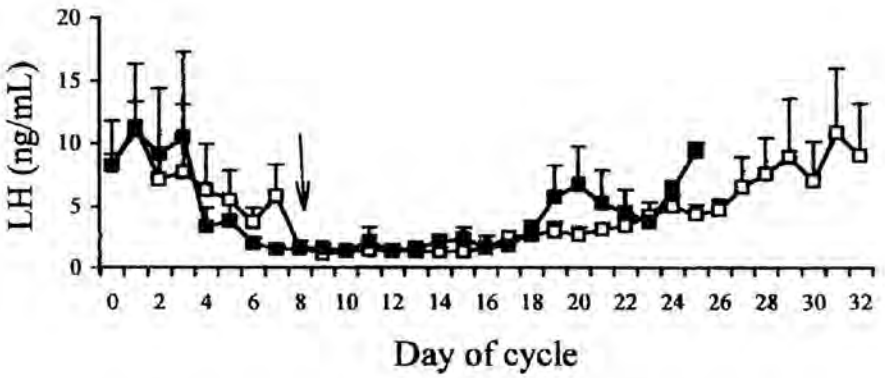

Figure 4. Mean (± SEM) concentrations of LH in 4 mares during a control (solid squares) and GnRH antagonist treatment (open squares) cycle. Arrow shows day of treatment.

lower (P=0.12) on Day 9 in the treatment cycle (1.3 ± 0.4 ng/mL) than in the control cycle (1.9 ± 0.5 ng/mL). On Day 8, 2 mares, and on Day 9, one mare had one high-amplitude pulse of LH during the control cycle, whereas none of the mares had high-amplitude LH pulses on either day during the treatment cycle.

In 1 of the 8 cycles (treated + control) studied there were two peaks of estradiol—the first in early diestrus and the second at estrus. In the remaining cycles the estradiol surge occurred at estrus. The surge was significantly (P=0.01) delayed in the treatment cycle (Day 28.8 + 4.6) compared with the control (Day 19.0 ± 1.4) cycle (Figure 5). Mean daily concentrations after antagonist treatment tended (P=0.08) to be lower in the treatment cycle (3.6 ± 0.4 pg/mL) than in the control cycle (7.5 ± 1.4 pg/mL).

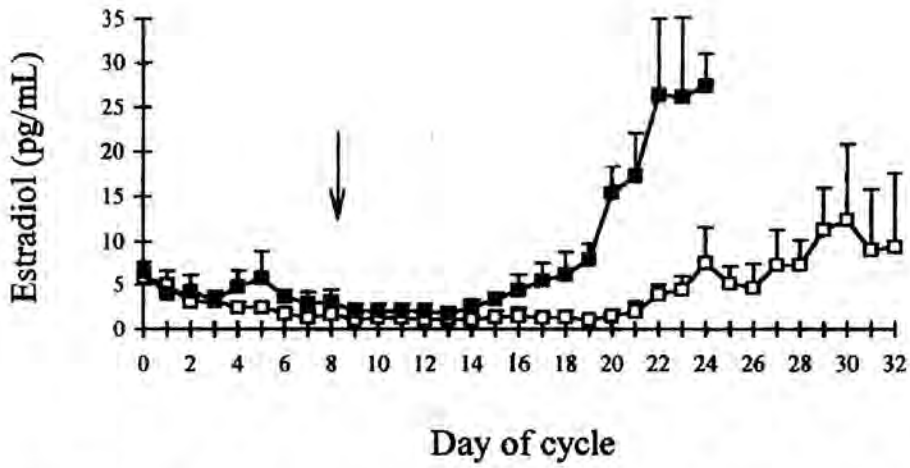

Figure 5. Mean (± SEM) concentrations of estradiol in 4 mares during a control (solid squares) and GnRH antagonist treatment (open squares) cycle. Arrow shows day of treatment.

DISCUSSION

Treatment of diestrous mares with a potent GnRH antagonist resulted in attenuation of progesterone secretion, indicating a role for LH in support of the mare CL. Although this occurred in the apparent absence of a decline in plasma concentrations of LH, it should be noted that in these mares, diestrous basal LH concentrations were very low and changes may have been too subtle to detect with our radioimmunoassay. Confirmation that the treatment reduced pituitary gonadotropin output was obtained from the ovariectomized mares, in which the initial LH and FSH concentrations were considerably higher than in the intact mares Treatment with GnRH antagonists in other species usually results in profound suppression of LH secretion within 4 hours of administration (5, 18, 39). However the long half-life of LH in the mare (approximately 5 hours; 19) compared with less than 1 hour in other species meant there was a delay in reaching very low serum concentrations after GnRH-antagonist treatment. Pituitary release was suppressed for a prolonged period of time in ovariectomized mares after a single subcutaneous injection of antagonist, suppression of LH being greater than that of FSH.

High-amplitude pulses of LH were not measured in any of the cyclic mares after antagonist treatment. Between Days 4 and 12 of diestrus, the mean frequency of LH pulses was found to be only 1.6 per day (21), which may explain why our sampling regimen failed to detect pulses in one of the control cycles. However the immediate drop in progesterone after GnRH-antagonist treatment in the present study indicates the importance of pulsatility of LH secretion in the maintenance of CL function in the mare. Interestingly, episodic secretion of progesterone was maintained on Days 8 and 9 in mares treated with antagonist (data not shown) despite the absence of high-amplitude LH pulses. This confirms the lack of correlation found between LH and progesterone pulses in mares (31, 38) and suggests the presence of an autonomous pulse-generating system within luteal tissue as described in the cow (35) and ewe (25). It is thought that FSH does not act as a luteotropin because of the low level of binding of FSH to the equine CL (37), and therefore it is unlikely that the effect seen on progesterone concentrations was caused by reduction in FSH concentrations after treatment.

The established mode of action of the GnRH antagonist is to block the GnRH receptors of the pituitary selectively (5). It is unlikely that the antagonist blocked a direct luteotropic action of GnRH on the CL. Receptors for GnRH have been found in the rat CL (6), but results from other species indicate that GnRH probably does not have a direct effect at the ovarian level (4, 14).

Diameter of the CL after antagonist treatment tended to be less than in the control cycle. Similarly, in a previous study, mares treated with antiserum against LH had CL that were lighter in weight than those of control mares (33). After GnRH-antagonist treatment, progesterone concentrations had fallen to relatively low levels by 48 h after treatment. After PGF-2α treatment, progesterone decreases to less than 1 ng/mL within 48 h (29). Therefore the timescale was similar to PGF-2α-induced luteolysis, although with the GnRH antagonist, luteolysis was incomplete. Complete luteolysis did not occur significantly earlier in the treatment cycle than in the control cycle. By contrast, administration of Antarelix™ to marmoset monkeys on Day 9 of the luteal phase resulted in luteolysis within 24 hours (40). The failure of the antagonist to cause luteolysis in mares indicates that progesterone secretion was supported either by basal LH concentrations or by some other luteotropin.

It has been reported that two broad surges of FSH occur at 10- to 12-day intervals during the estrous cycle of the mare (10). One of the surges occurs during late estrus and early diestrus and the other in late diestrus. However there is little consistency in patterns among individual animals (16), and no late estrous surge was seen in any of our mares. A recent paper by Irvine and coworkers (21) shows that sampling one time per day for FSH concentrations may be misleading because of the markedly pulsatile secretion. In general FSH concentrations in our mares were low throughout estrus and elevated in diestrus. The late diestrous surge of FSH in the mare appears to be associated with selection and growth of a dominant follicle (1, 2, 11, 26). In other species the suppressive effect of GnRH-antagonist treatment on FSH secretion is less than for LH (5). However antagonist treatment appeared to delay the diestrous rise in FSH in the intact mares as previously reported (30). Suppression of FSH and/or LH by administration of either charcoal-stripped follicular fluid or antiserum against a crude gonadotropin preparation, active immunization against LHRH (36) or chronic GnRH agonist treatment inhibits follicular development in the mare (1, 32). In the present study the delay in appearance of the preovulatory increase in LH and the diestrous increase in FSH in the treatment cycle may

account for the retarded follicular growth. However, precise information on gonadotropic control of folliculogenesis in the mare is lacking, and we did not study pulsatility of gonadotropin release in the preovulatory period. Equine follicles can develop to preovulatory size under a variety of FSH patterns (16). However, it seems that maturation of the follicle requires LH or FSH plus LH, and it is thought that LH is required for the prolonged maturation of the previously primed preovulatory follicle during estrus, when FSH concentrations are low (10). The results from our study where follicular development was significantly delayed by GnRH-antagonist treatment indicate a role for gonadotropins in preovulatory follicular growth and maturation.

The mare that failed to show a sustained drop in progesterone after antagonist treatment had a large 40-mm anovulatory follicle from Day 5 after ovulation that showed ultrasonographic evidence of luteinization and persisted until the end of diestrus. This mare also had very high concentrations of progesterone during this cycle compared with the other mares, suggesting that the anovulatory follicle was indeed producing progesterone. Failure of this mare to respond to the GnRH antagonist indicates that this luteinized follicle was not under the same luteotropic control as a CL. The etiology and incidence of formation of luteinized anovulatory follicles in nonpregnant mares is not known, but it would appear that either there is insufficient gonadotropic stimulation to induce ovulation or the follicle fails to respond to gonadotropin stimulation.

In conclusion the results indicate that high-amplitude LH pulses are involved in maintaining progesterone production by the equine CL. The delay in follicle growth and ovulation in mares treated with a GnRH antagonist confirms a role for gonadotropins in preovulatory follicle development.

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BUTTERWORTH HEINEMANN

LH AND PROGESTERONE CONCENTRATIONS DURING DIESTRUS IN THE MARE AND THE EFFECT OF hCG

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ABSTRACT

The temporal association between circulating concentrations of LH and progesterone was studied by collecting blood samples from 4 mares at 15-min intervals for 12h on Days 3, 8 and 13 of diestrus. Luteinizing hormone appeared to be secreted in an episodic fashion on Days 8 and 13, but on Day 3 of the cycle discrete pulses of LH were detected in only 1 mare. Episodic progesterone secretion was detected on all days of sampling. No correlation was observed between LH and progesterone concentrations. Intravenous administration of 1,500 IU hCG to mares resulted in significantly elevated circulating concentrations of progesterone in 3 of the 4 mares. These results suggest that exogenous LH can be luteotrophic for the mare corpus luteum but that there was little temporal relationship between endogenous circulating LH and progesterone in peripheral blood.

Key words: LH, progesterone, hCG, mare

INTRODUCTION

Secretion of progesterone by the corpus luteum (CL) is essential for the establishment and maintenance of early pregnancy in the mare. In other species there is strong evidence that LH plays an important role in support of the CL (4). However, there is little information on control of secretion and production of progesterone by the mare CL. Receptors for LH have been identified in equine luteal tissue (32), and their numbers have been reported either to increase (30) or to remain constant (7) during diestrus. Treatment of mares with an antiserum raised against pituitary extract resulted in the mares developing CL of lower weight than the control mares. These CL also appeared, macroscopically, to be regressing prematurely (27,28), thus providing

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some evidence for the involvement of pituitary support. Addition of LH or hCG to equine luteal cells in vitro has either stimulated progesterone secretion (20) or had no effect (7, 9, 35). In another study, intramuscular administration of hCG to mares in early diestrus resulted in increased plasma concentrations of progesterone several days later (20), but it is not clear whether the increase in progesterone was caused by luteinization and/or ovulation of ovarian follicles, or by direct stimulation of the CL. In the present study, we 1) investigated the temporal relationships between plasma concentrations of LH and progesterone in blood samples collected at frequent intervals during diestrus, and 2) determined the effect of intravenous hCG administration on progesterone production in vivo by the mare CL.

MATERIALS AND METHODS

Four pony mares between 3 and 15 years of age and weighing 213 to 280 kg were used. Ovulation was detected by ultrasonography, and the day of ovulation was designated as Day 0. On Days 3, 8 and 13 of the cycle, an indwelling catheter was placed aseptically in the jugular vein under local anaesthesia. Catheter patency was maintained by flushing with heparinized saline. Blood samples were withdrawn into heparinized evacuated tubes at 15-min intervals for 12 h between approximately 07.30 and 19.30 h. During a further cycle, blood samples were collected at 15-min intervals for 12 h on Day 8, and 1500IU hCG[a] were administered intravenously after 6 h. Blood samples were stored on ice until centrifuged at 2,000 g for 10 min. Plasma was stored at -20°C until assayed for progesterone and LH concentrations.

Progesterone Assay

Progesterone concentrations were determined in unextracted plasma using a radioimmunoassay kit[b] which employed an ^{125}I-progesterone tracer. Standards were prepared in plasma from an ovariectomized mare. The main (>0.5%) cross-reactivities of the antiserum were with 20α-dihydroprogesterone (5.4%), desoxycorticosterone (3.8%), corticosterone (0.7%) and 17-hydroxyprogesterone (0.7%). The limit of detection of the standard curve was 0.25 ng/ml and the within- and between-assay coefficients of variation were 6.8 and 6.0%, respectively.

LH Assay

Luteinizing hormone was measured in the plasma by the method of Price *et al* (29). Purified equine LH (E98A) for standards was supplied by Dr H. Papkoff, University of California. Ovine LH for radioiodination was obtained from NHPP, NIDDK, NICHHD and USDA, Rockville, MD, USA. The antiserum for the LH assay was donated by Dr D.L. Thompson, Louisiana State University, and was raised in rabbits against equine chorionic gonadotrophin (33). Cross-reactivities of the antiserum with equine FSH and with thyroid stimulating hormone were 2.4 and 3.8%, respectively. The limit of detection of the assay was 1.25 ng/ml using 200-µl samples. When different volumes of a diestrous plasma sample were assayed (200, 100, 50 and 25 µl), the regression equation of expected versus measured

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[a] Chorulon, Intervet UK Ltd, Cambridge, UK.

[b] ICN Biomedicals Inc, Costa Mesa, CA, USA

concentration was y = - 0.27+1.19x with a coefficient of correlation of 0.99. Within- and between-assay coefficients of variation were 7.5 and 14.7%, respectively.

Statistical Analyses

Luteinizing hormone and progesterone pulses were identified by the Munro pulse analysis programme.[c] The identified pulses were also checked by subjective criteria from visual inspection of the data. Differences in pulse amplitude and frequency between the stages of diestrus were determined by a one-way analysis of variance with least significant difference between mean comparisons where appropriate. Correlation between secretory episodes of LH and progesterone required that the uptake of a progesterone pulse followed within 60 min of the upstroke of an LH pulse (31). Differences in mean progesterone concentrations, pulse amplitudes and frequencies were compared before and after hCG administration by a paired t-test. For each individual mare, progesterone concentrations were also compared before and after hCG administration by the Student's t-test.

RESULTS

Examination of progesterone profiles showed that 2 of the mares were undergoing luteolysis on Day 13 and their results were not analyzed for this day. Progesterone and LH appeared to be secreted in an episodic manner during mid- and late diestrus. Progesterone also appeared episodic in early diestrus but a clearly defined LH pulse was detected in only one of the four mares. Figures 1, 2 and 3 show LH and progesterone profiles from representative mares. Mean progesterone concentrations and progesterone pulse amplitude were greater ($P<0.01$) in mid-diestrus than in early diestrus, but pulse frequency was not affected by cycle stage (Table 1). On Day 3 of diestrus, but not on Day 8, progesterone concentrations increased ($P<0.01$) over the 12 h sampling period in 3 of the 4 mares. There did not appear to be any temporal association between LH and progesterone episodic secretion in that the progesterone pulses occurred 3 to 9 times more frequently and were usually independent of LH pulses.

Administration of hCG (Figure 4) did not affect frequency of progesterone pulses but tended ($P = 0.1$) to increase mean pulse amplitude and mean concentrations (Table 2). When data from individual mares were compared, hCG administration significantly increased ($P<0.01$) progesterone concentrations in 3 of the 4 mares. During the control cycle (above) when hCG was not administered on Day 8, there were no significant differences in progesterone concentrations in the first 6 h of sampling compared with the second 6 h.

DISCUSSION

In the present study we have shown that distinct pulses of both LH and progesterone are detectable in peripheral blood during diestrus in the mare. However, episodic LH secretion was seen in only 1 of 4 mares early in diestrus. Pulses of LH in the peripheral circulation of the mare

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[c] Elsevier-Biosoft, Cambridge, UK.

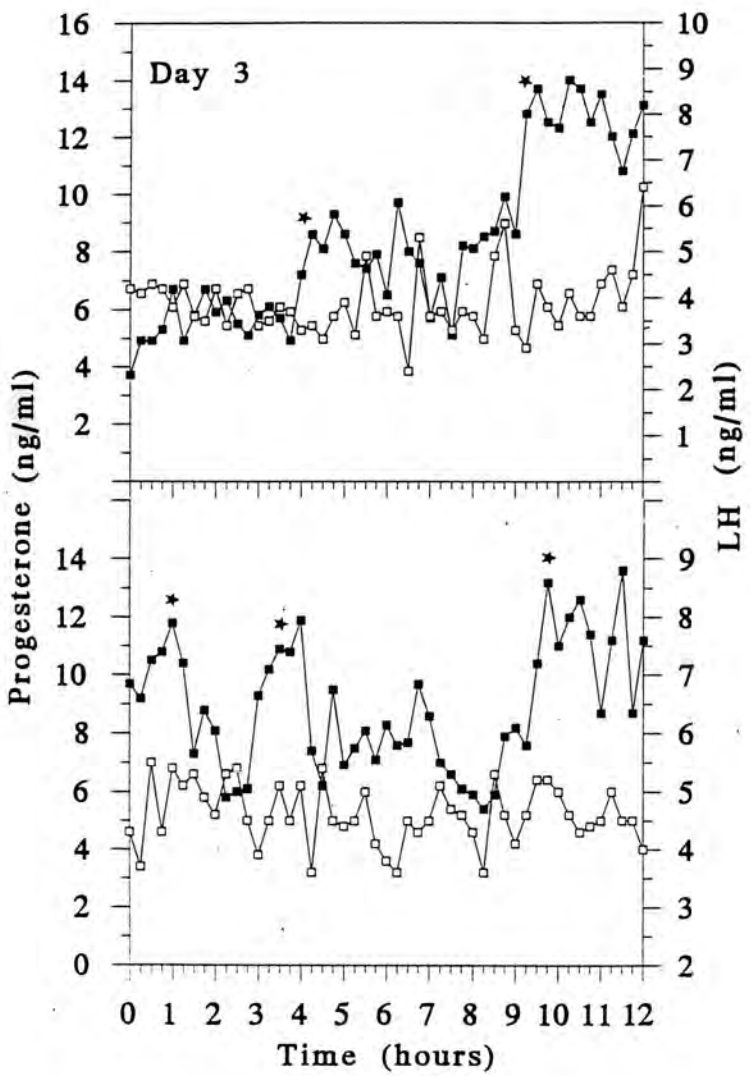

Figure 1. Profile of progesterone (closed squares) and LH (open squares) from 2 mares on Day 3 of diestrus. Pulses are indicated by asterisks.

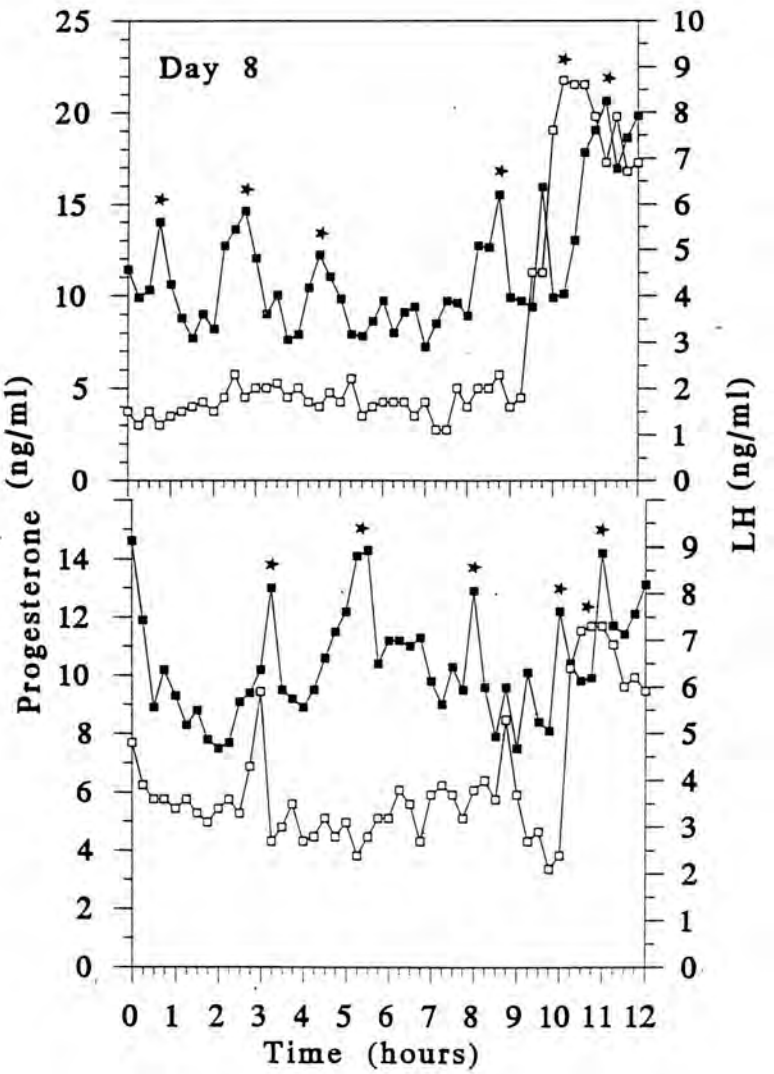

Figure 2. Profile of progesterone (closed squares) and LH (open squares) from 2 mares on Day 8 of diestrus. Pulses are indicated by asterisks.

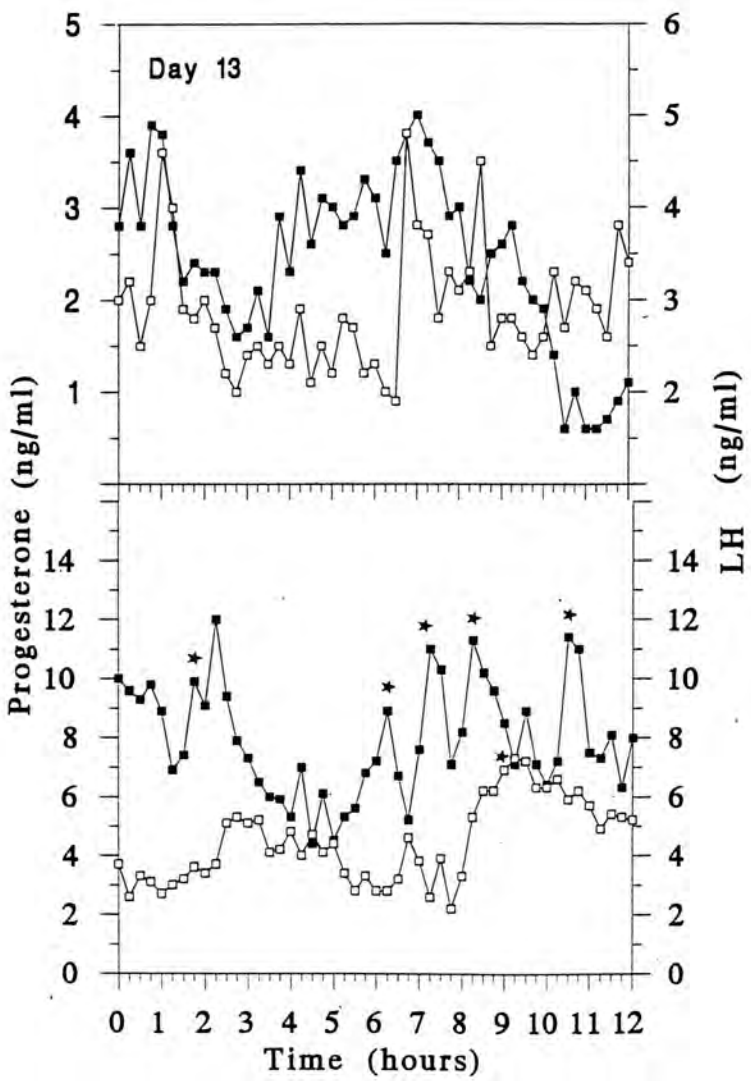

Figure 3 Profile of progesterone (closed squares) and LH (open squares) from 2 mares on Day 13 of diestrus. Pulses are indicated by asterisks. The top mare was undergoing luteolysis and her results were not analyzed.

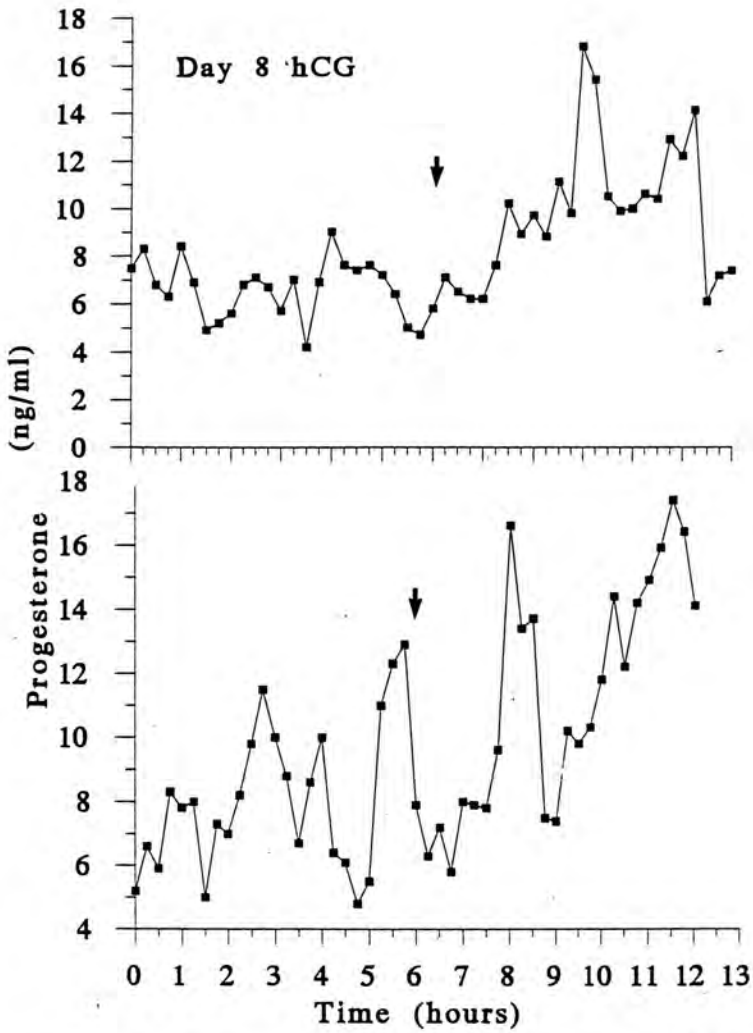

Figure 4. Concentrations of progesterone from 2 mares that received 1,500 IU hCG by the intravenous route on Day 8. The arrow represents the time of hCG administration.

Table 1. Relationship between concentrations of LH and progesterone (P4) in plasma during the estrous cycle of 4 mares

Day post ovulation	n	No of LH pulses per 12 hours	Mean LH concentration (ng/ml)	No of P4 pulses per 12 hours	Mean P4 concentration (ng/ml)
3	4	0.3±0.25	4.7±0.28	2.8±0.48	6.5±1.22[a]
8	4	0.8±0.25	3.4±0.34	3.8±0.25	13.2±1.69[b]
13	2	1.0±1.00	3.8±0.48	4.0±1.0	8.0±3.55

[a,b] $P<0.01$.

Table 2. Concentrations of progesterone in the plasma before and after administration of hCG to 4 mares on Day 8 of the estrous cycle

	Before hCG	After hCG
Mean progesterone pulse frequency (pulses/hours ± SEM)	0.29 ± 0.05	0.25 ± 0.06
Mean progesterone pulse amplitude (ng/ml ± SEM)	3.39 ± 0.58	5.29 ± 0.88
Mean progesterone concentrations (ng/ml ± SEM)	9.65 ± 2.21	12.63 ± 3.40

have previously been reported during diestrus (1, 15, 25) at a similar frequency to that in the present study. During the periovulatory period, it is accepted that pulses can only be detected in pituitary venous blood (2), presumably due to the low amplitude of pulses and the long half-life of equine LH (approximately 2 to 5 h; 16, 19), and this probably accounts for the failure to detect pulses in 3 of 4 mares on Day 3 after ovulation.

Peripheral concentrations of progesterone varied up to four-fold over a 12-h sampling period in the present study, with mean concentrations being significantly greater in mid-diestrus than in early diestrus. The higher mean concentration was primarily due to the greater amplitude of the progesterone peaks during mid-diestrus (5.4 ± 0.61 ng/ml) than during early (2.2 ± 0.30 ng/ml) diestrus. Reports of progesterone measured in blood samples collected twice daily during diestrus have shown large differences in concentration between the 2 samples (8, 24, 26), and 3- and 15-min sampling intervals have revealed a pulsatile release of progesterone (2, 26). Although it has previously been suggested that progesterone may exhibit a circadian rhythm (24), neither the study by Perkins et al. (26) nor the present study found any evidence of a circadian rhythm in progesterone concentrations. On Day 3 of diestrus, progesterone concentrations were higher at the end of the sampling period than at the start, but this was presumably due to maturation of the immature CL as it attained its maximum functional capacity. The wide fluctuations in progesterone concentrations measured in our study show that a single daily blood sample from a mare is unlikely to provide an accurate indication of luteal function. There is some evidence based on measurement of progesterone in once-daily blood samples that luteal inadequacy may cause early embryonic loss in some mares (10). However, our results and those of others (25) suggest that such data should be regarded with caution.

We did not detect any correlation between secretory episodes of LH and progesterone in peripheral blood. Evans (13) and Perkins et al. (25) similarly did not find any temporal correlation between LH and progesterone secretion patterns in the mare. In the present study, progesterone peaks were detected at much higher frequency than LH, and it has been suggested in a study in women that the CL may have an inherent pulsatile pattern of progesterone release which may then be modified by the action of LH (31). Alternatively, changes in blood flow from the CL or variations in metabolic clearance rate of progesterone may account for the observed episodic pattern. Perkins et al. (25) have suggested that LH may be involved only in the formation of the mare CL and that once the CL is mature an uncoupling may occur between progesterone and LH release. Another important factor in control of CL function is the heterogeneity of equine LH that results in anomalies in bioactive versus immunoreactive LH. It is thought that α subunit oligosaccharides, which are primarily attached to Asn^{56}, are important in the recognition of equine LH by target cells (17), and therefore the intrinsic polymorphism of LH may account for the apparent lack of relationship between immunoreactive LH and progesterone concentrations, and the lack of responsiveness of luteal cells to LH stimulation in vitro (7,35). In other species there are conflicting data on the association between these hormones. In cattle (34), rhesus monkeys (11), women (14) and ewes (4) pulses of LH and progesterone in the blood have been reported to be closely associated, whereas other studies in ewes (22) and rhesus monkeys (18) have failed to confirm a temporal association between LH and progesterone. Furthermore, when pulsatile LH secretion was suppressed by administration of a GnRH antagonist to ewes, progesterone secretion by the CL was not affected, indicating that in the ewe episodic secretion

of progesterone may be independent of LH pulses (23). However, this finding was not supported by similar antagonist studies in the human (21) and rhesus monkey CL (18). It is difficult to compare results from other species with those of the mare due to the differences in hormone kinetics in the mare, characterized by the elevation in LH concentrations for several days around ovulation due to the long half-life of LH and the extremely rapid rise in progesterone concentrations after ovulation (12). These differences could account for the relative parity in LH secretion characteristics over the phases of diestrus in our study compared with the findings from other species, in which the pattern of LH secretion alters during diestrus (11, 34). Despite failing to detect a temporal relationship between LH and progesterone, we showed that in 3 of 4 mares administration of hCG caused an increase in blood progesterone concentrations. Thus we have provided some evidence that an exogenous LH-like substance can modify CL function.

In our present study progesterone pulses occurred more frequently than LH pulses. In cows, FSH pulses occurred more frequently than LH pulses in diestrus, and almost all of the FSH pulses were followed by a pulse of progesterone. This suggested that FSH was the principle hormone which stimulated progesterone secretion (18). However, in the mare, more than 90% of the FSH and LH pulses are reported to occur coincidentally (3), and the mare CL has far fewer receptors for FSH than for LH (32). Furthermore, treatment of early pregnant mares with charcoal-extracted equine follicular fluid reduced circulating concentrations of FSH but had no effect on plasma progesterone concentrations (5). It seems unlikely, therefore, that FSH has a central role in the control of luteal steroidogenesis in the mare. The role of other luteotrophic factors, including paracrine and autocrine control, remains to be investigated. We have recently shown that the mare possesses particulate binding sites, distinct from classical progesterone receptors, in luteal cell-surface membranes (6). Thus there exists a local mechanism whereby progesterone could have an autocrine/paracrine role in controlling its own secretion.

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Isolation of cell populations from the mare corpus luteum: comparison of mechanical and collagenase dissociation

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Corpora lutea were obtained from mares at days 3, 10 and 14 after ovulation, and examined histologically. The morphology of isolated luteal cells obtained by either mechanical or collagenase dissociation of the tissue was examined and the cells stained to detect the steroidogenic enzyme Δ^5, β-hydroxysteroid dehydrogenase. The ratio of large:small cells was significantly higher for cells obtained from mechanically dissociated luteal tissue than for cells obtained by collagenase dissociation ($P < 0.01$). Cells obtained by both mechanical and collagenase dissociation secreted progesterone, although neither cell population responded to exogenous gonadotrophin with an increase in progesterone secretion. Homogenates of equine luteal tissue bound ^{125}I-labelled human LH with high affinity and specificity, and the specific activity and binding affinity of luteal LH receptors did not change significantly from day 3, to days 10 and 14 after ovulation. However, mechanically dissociated cells on days 10 and 14 bound significantly more LH than did collagenase-dissociated cells on these days ($P < 0.05$). These results indicate that (i) collagenase dissociation of mare luteal tissue yields a population of cells that is unrepresentative of the corpus luteum, and (ii) the mare corpus luteum is not responsive to LH *in vitro* at the stages examined.

Introduction

The functional control of the corpus luteum in humans and domestic animals has been reviewed recently by Baird (1992) and Wiltbank and Niswender (1992). In humans and domestic animals, the primary luteotrophin is LH. *In vitro*, LH enhances the secretion of progesterone from porcine luteal slices (Watson and Leask, 1975), bovine luteal tissue (Condon and Black, 1976) and from dissociated luteal cells from pigs, cattle and sheep (Lemon and Loir, 1977; Pate and Condon, 1982; Hoyer *et al.*, 1988, respectively). The corpus luteum of these domestic species is composed of two distinct types of steroidogenic cell, which are thought to be derived from the follicular granulosa and theca interna layers. These cells differ in their secretory function: large luteal cells are thought to be derived from granulosa cells, secrete high basal amounts of progesterone and are unresponsive to LH; small luteal cells are thought to derive from thecal cells, have a low basal secretion of progesterone, but respond to LH with a dramatic increase in steroid secretion (Koos and Hansel, 1981; Fitz *et al.*, 1982). Many of the properties of isolated luteal cells have been defined using isolated cell preparations obtained by collagenase dispersion (Simmons *et al.*, 1976); however, it is known that cell damage and specific loss of large cells can occur during this procedure (O'Shea *et al.*, 1989; Nelson *et al.*, 1992).

Although much is now known about the control of the corpus luteum in other domestic species, relatively little is known about the control of the mare corpus luteum. Oestrous cycle disorders such as dioestrous ovulations and prolonged retention of the corpus luteum in unmated mares are not uncommon. Furthermore, the mare is unusual in that secondary corpora lutea develop during pregnancy, suggesting that the regulatory mechanisms of the mare corpus luteum may differ from those in other species. In contrast to other domestic species (see above), only the granulosa cells are thought to contribute to the fully functional mare corpus luteum (Van Niekerk *et al.*, 1975). Although collagenase dispersion of equine luteal tissue yields cells of different sizes (Kelly *et al.*, 1988; Watson and Sertich, 1990), the steroidogenic potential of these cell populations is unclear, since increased progesterone secretion was not observed when LH was added to slices of equine luteal tissue (Condon *et al.*, 1979), and the effect of gonadotrophin treatment on collagenase-dispersed equine luteal cells has been reported as either stimulatory (Kelly *et al.*, 1988) or as having no effect (Watson and Sertich, 1990). However, the lack of response to LH was not due to the absence of LH receptors (Stewart and Allen, 1979; Roser and Evans, 1983).

The objective of this study was therefore to characterize the mare corpus luteum in terms of the different (steroidogenic) cell

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types present, the presence of LH receptors throughout the luteal phase, and the steroidogenic responsiveness of isolated luteal cell types. Preliminary studies of the cell populations obtained by mechanical and collagenase dissociation of the mare corpus luteum led us to carry out a more detailed comparison of the two cell-dispersion techniques.

Materials and Methods

Materials

hCG (Chorulon; 1500 iu hCG per ampoule) and pregnant mares' serum gonadotrophin (PMSG; Folligon; 1500 iu per ampoule) were purchased from Intervet Laboratories, Cambridge, UK. Equine LH (eLH E263B) and equine FSH (E276B) were obtained from H. Papkoff, San Francisco, CA. Mouse epidermal growth factor (mEGF) was the generous gift from K. Brown, Babraham, Cambridge, UK, and the GnRH agonist buserelin (D-[(tBu)Ser^6] GnRH ethylamide) was the kind gift of J. Sandow, Hoescht AG, Frankfurt. Highly purified hCG (CR-125), ovine LH, ovine FSH, and ovine and human prolactin were obtained from the Hormone Distribution Officer, NIAMDD, Bethesda, MD. ^{125}I-Labelled hLH (100 μCi μg^{-1}) was purchased from K. Ferguson (Chelsea Hospital for Women, London). Collagenase D (0.34 U mg^{-1}) was purchased from Boehringer Mannheim (East Sussex). All other materials were obtained from Sigma Chemical Co. (Poole).

Animals

Twelve pony mares, 250–350 kg and 3–15 years of age, were used in this study. Throughout the spring and summer, the mares exhibited normal patterns of oestrus and ovulation. During oestrus, follicular growth was monitored daily by real-time ultrasonography per rectum until ovulation was detected (day of ovulation = day 0). The ovary containing the corpus luteum was removed 3, 10 or 14 days after ovulation by colpotomy (Watson and Sertich, 1990). Acepromazine (0.05 mg kg^{-1}; C-Vet Ltd, Bury St Edmunds), rimifidine (0.05 mg kg^{-1}; Boehringer Ingleheim Ltd, Bracknell) and butorphanol (0.05 mg kg^{-1}; Willow Francis, Crawley) were administered i.v. to induce sedation and analgesia. After a subsequent ovulation the remaining ovary was removed on day 3, 10 or 14 of dioestrus. A blood sample was collected by jugular venepuncture immediately before surgery, and plasma was stored at −20°C until it was analysed for progesterone.

Processing of the corpus luteum

After surgery, the corpus luteum was removed from the ovary; a piece of tissue was snap-frozen immediately in liquid nitrogen for LH binding analysis; and 2–3 wedges of tissue were placed in Bouin's fixative for histological analysis. Paraffin wax sections were prepared and stained with haematoxylin and eosin. Cell diameters were measured by randomly selecting six fields per slide and estimating the diameters of all the cells in each field using an ocular micrometer. At least four different sections and 200 cells for each stage were measured.

Isolation and dissociation of luteal cells

The remainder of the tissue was minced into pieces approximately 1 mm^3 and placed in a 50 ml centrifuge tube (Falcon, Becton Dickinson UK Ltd, Oxford) in 30 ml of Hank's balanced salts solution (without divalent cations) buffered with 20 mmol Hepes l^{-1} (buffered HBSS; Flow Laboratories, Irvine). Cells were mechanically dissociated from the tissue by gently inverting the tube 10–15 times. The suspension was passed through a 100 μm stainless steel mesh, the cells were collected by centrifugation (400 *g*, 10 min) and resuspended in buffered HBSS. One piece of the remaining tissue was placed in Bouin's fixative for histological examination. The rest was subjected to enzymatic digestion by the method of Simmons *et al.* (1976), but using collagenase D (0.34 U mg^{-1}). (This collagenase preparation was selected because it has a relatively low tryptic activity (< 0.1 U mg^{-1}) compared with other commercially available collagenase preparations, and is recommended for studies where functionality and surface protein integrity are important considerations.) The tissue was added to dissociation medium (buffered HBSS containing divalent cations, 10% fetal calf serum, 0.02% DNAse, 100 U penicillin ml^{-1}, 100 μg streptomycin ml^{-1} and 1 mg collagenase D ml^{-1}) and incubated in a shaking water bath at 37°C for 45 min (3 ml g^{-1} tissue). After digestion, the cells were washed twice with buffered HBSS and collected by centrifugation (400 *g*, 10 min). Large and small cells in both mechanically and collagenase-dissociated cell preparations were counted using a haemocytometer. Cell viability was assessed using fluorescein diacetate (Rotman and Papermaster, 1966). Cytospin preparations of both mechanically dissociated and collagenase-dissociated cells were stained with Diff-Quik™ (Baxter, Thetford), or for the presence of Δ^5, β-hydroxysteroid dehydrogenase (3β-HSD) using the method of Payne *et al.* (1980).

Culture of luteal cells

Mechanically dissociated and collagenase-dissociated cells were suspended in culture medium (M199 and 20 mmol Hepes l^{-1} containing 100 u penicillin ml^{-1} and 100 μg streptomycin ml^{-1}, pH 7.2) at a concentration of 10^5 cells ml^{-1}, and 200 μl aliquots were added to multiwell tissue culture dishes (Falcon). Equine LH (10–500 ng ml^{-1}) or hCG (0.1–100 iu ml^{-1}) were included in the incubation medium. The cells were incubated at 37°C under a humidified atmosphere of 5% CO_2:95% air for 2–6 h or for 20 h. After incubation, the medium was collected, centrifuged at 400 *g* for 10 min to remove the cells and the supernatant stored at −20°C until assayed.

Progesterone assay

Progesterone was assayed in unextracted plasma or culture medium by a double-antibody radioimmunoassay using a specific antiserum provided by R. Webb (The Roslin Institute, Midlothian), as described by Webb *et al.* (1985). The limit of detection of the assay was 0.1 ng ml^{-1} and the intra-assay and interassay coefficients of variation were 5.3% and 7.7%, respectively. Standards were prepared in equine plasma or in culture medium and showed good parallelism. The

principal crossreactivities of the antibody (> 1.0%) were with 11α-hydroxyprogesterone (22.67%), 11-deoxycorticosterone (7.71%), 20α-hydroxy-4-pregnen-3-one (2.15%), 5-pregn-3β-ol-20-one (1.48%) and 5α-pregnane 3,20-dione (1.42%) (C. Ashworth, personal communication). Progesterone concentration was adjusted for the number of cells and secretion was normalized for differences in the duration of incubation.

LH binding assay

Homogenates of equine luteal tissue or sonicated cells were incubated in triplicate with Tris–acetate buffer (40 mmol l^{-1}, pH 6.5), 0.5% BSA and 100 000 c.p.m. [^{125}I]-labelled hLH at 20°C for 16–20 h (Bramley *et al.*, 1987). Nonspecific binding was assessed in duplicate by the inclusion of 50 iu hCG. After incubation, tubes were chilled on ice, bound and free hormone were separated by polyethylene glycol precipitation, and the amount of ^{125}I associated with the pellet was determined at an efficiency of 75% in a multiwell Packard Crystal gamma counter. The difference between total binding and binding in the presence of excess unlabelled hormone represented specific binding. A preparation of sheep corpora lutea homogenate with a binding capacity of 5.1 fmol ^{125}I-labelled hLH μg^{-1} DNA was included as an internal standard in each assay (interassay coefficient of variation was 4.5%; $n = 3$). The specificity of binding was assessed by measuring [^{125}I]-labelled hLH binding in the absence, or in the presence, of increasing concentrations of various hormones, and the concentration of each hormone required to reduce specific hLH binding by 50% (IC_{50}) was calculated.

Scatchard analysis

Equilibrium constants (K_a) and maximum binding capacity were calculated by Scatchard analysis (Scatchard, 1949) of binding isotherms for ^{125}I-labelled hLH binding (45–900 pg) to homogenates of corpora lutea tissue obtained at different stages of the luteal phase. Binding affinity and receptor concentration were calculated by linear regression analysis, and the number of binding sites was corrected for homogenate DNA concentration as a measure of cell content.

DNA assay

DNA was measured fluorimetrically using the method of West *et al.* (1985).

Statistical analyses

All values are expressed as means ± SEM. The significance of differences between means was assessed using Student's *t* test. Progesterone secretion by cells from different stages of dioestrus and LH binding to luteal tissue homogenates at different stages of dioestrus were compared by analysis of variance (ANOVA). Values of $P < 0.05$ were considered to be significant.

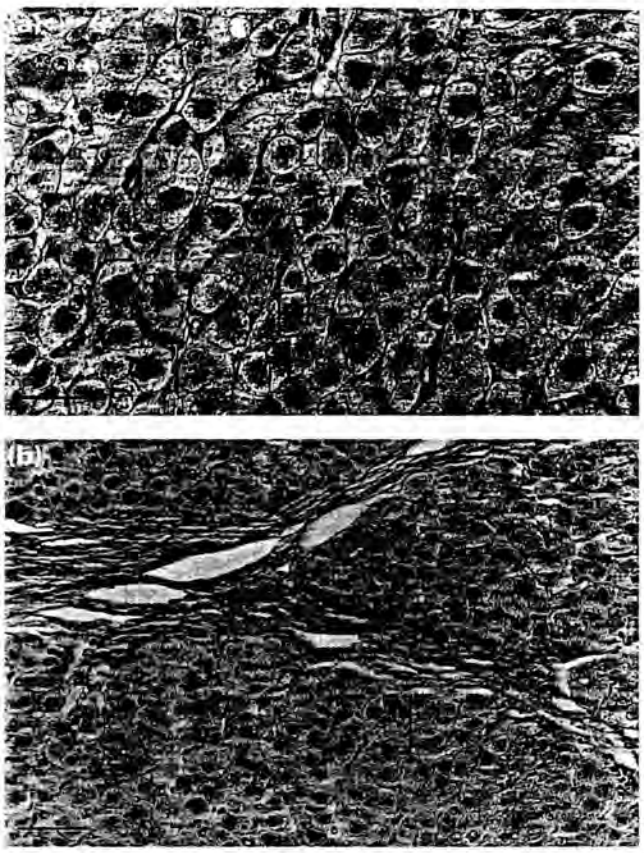

Fig. 1. Equine corpus luteum at mid–late cycle (day 10) showing (a) large cells with some small cells interspersed (scale bar represents 65 μm), and (b) trabeculae containing small elongated cells with darkly staining nuclei. Scale bar represents 140 μm.

Results

Plasma progesterone

Mean plasma progesterone concentrations immediately before surgery increased significantly ($P < 0.01$) between day 3 (6.3 ± 0.6 ng ml^{-1}; $n = 6$) and day 10 (11.6 ± 1.3 ng ml^{-1}; $n = 13$) but had declined by day 14 (8.6 ± 2.2 ng ml^{-1}; $n = 4$), although this was not a significant result ($P > 0.1$).

Histology

Three days after ovulation the corpus luteum contained substantial amounts of fibrin and blood, and in some cases still appeared to be undergoing organization in many areas. Large and small cells at this stage were 26.3 ± 1.2 μm and 9.4 ± 1.4 μm in diameter, respectively. Mid- and late-cycle corpora lutea appeared to be highly organized and consisted of areas containing large cells interspersed with small cells (Fig. 1a). These corpora lutea displayed distinct trabeculae of extracellular matrix, which contained much of the vasculature of the corpus luteum, along with some small cells (Fig. 1b). Within the trabeculae, small, elongated, nonvascular cells with darkly staining nuclei were observed. Small cells embedded

Table 1. Ratio of large:small cells (% ± SEM) obtained by mechanical and collagenase dissociation of equine corpora lutea from different stages of dioestrus; and the cell yield (mean ± SEM) from dissociation procedures

Procedure	Stage of dioestrus (days)		
	3	10	14
Mechanical dispersion			
Large:small cell ratio (%)	62.5 ± 7.2	55.3 ± 5.9	45.7 ± 4.2
Cell yield (10^{-7} g^{-1} wet mass)	2.2 ± 1.0	3.1 ± 1.2	1.4 ± 0.5
Collagenase dispersion			
Large:small cell ratio (%)	18.0 ± 5.5	17.1 ± 5.5	10.3 ± 1.3
Cell yield (10^{-7} g^{-1} wet mass)	1.5 ± 0.9	1.4 ± 0.6	0.6 ± 0.4

n = at least 4 for each treatment day^{-1}.
Large:small ratio was significantly lower for cells obtained by collagenase dispersion compared with those obtained by mechanical dispersion on each day ($P < 0.01$).
Cell yields from the two dispersion methods were not significantly different at any stage ($P > 0.1$).

within a matrix were also observed around the periphery of the corpus luteum.

By day 10, the mean diameter of large cells had increased to 36.2 ± 1.3 μm, which was significantly different from that on day 3 ($P < 0.01$). These cells had pale-staining cytoplasm with a round nucleus containing one or more darker staining nucleoli. In some areas, the large cells were highly vacuolated. The small cells were 11.4 ± 0.8 μm in diameter and contained darkly staining nuclei. By day 14, the morphology of the corpus luteum was not markedly different from that on day 10, except that there were signs of disruption of structural integrity, with 'empty' areas throughout the tissue.

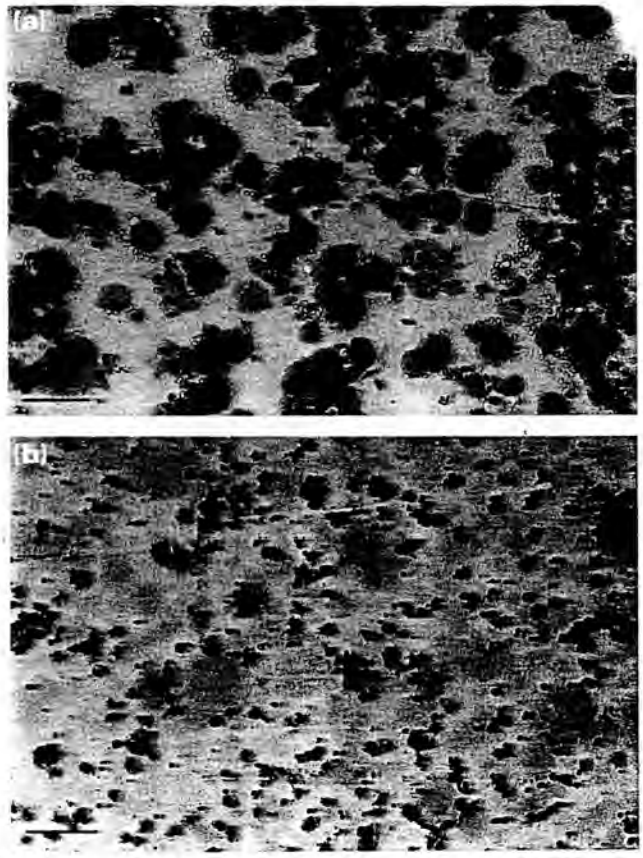

Fig. 2. (a) Mechanically dissociated cells and (b) collagenase-dissociated cells from the equine corpus luteum on day 10 after ovulation. Scale bars represent 100 μm.

Dissociation of luteal tissue

Although cell viability was similar for mechanically dissociated (80–90%) and collagenase-dissociated luteal cell preparations (70–80%), collagenase dispersion yielded significantly fewer large cells than did mechanical dissociation ($P < 0.01$) at all stages of the luteal phase (Table 1). Histological examination of the luteal tissue remaining after mechanical dissociation showed that it was indistinguishable from intact luteal tissue, suggesting that only a small proportion of the total large cell complement had been removed by mechanical dispersion.

Cytospin preparations of cells obtained by mechanical (Fig. 2a) and collagenase (Fig. 2b) dissociation showed evidence of greater cellular damage following collagenase treatment. Staining for 3β-HSD (Fig. 3) indicated that most activity occurred in large cells (although not all large cells stained). There was no obvious correlation between stage of the luteal phase and the degree of 3β-HSD staining.

Progesterone secretion

Cells obtained by collagenase dissociation on days 3 and 14 secreted more ($P < 0.01$) progesterone than did cells obtained by mechanical dissociation of the same tissue. However, there was no significant difference in progesterone secretion between the two cell preparations from tissue obtained on day 10 (Table 2). Isolated cells from luteal tissue at days 10 and 14 after ovulation secreted significantly more progesterone ($P < 0.01$) than did cells isolated on day 3 (Table 2). The addition of a range of concentrations of eLH or hCG did not alter progesterone secretion by either mechanically dissociated

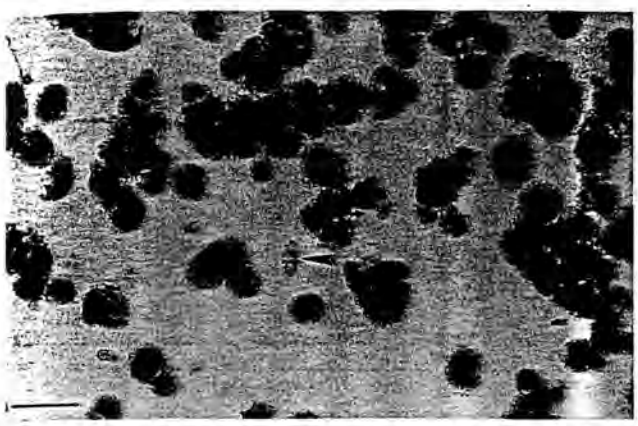

Fig. 3. Mechanically dissociated day 10 equine luteal cells stained with Δ^5, β-hydroxysteroid dehydrogenase (β-HSD) (arrow indicates small cells staining negatively for β-HSD). Scale bar represents 100 μm.

or collagenase-dissociated cells cultured over 2–6 h or 20 h (Table 2).

LH binding studies

Specific binding of radiolabelled hLH increased linearly with increasing equine luteal homogenate concentration (Fig. 4a), and was dependent on the pH (Fig. 4b) and metal ion concentration of the incubation buffer (data not shown), and on the temperature and duration of incubation (Fig. 4c). Scatchard analysis of hLH binding to equine corpora lutea (Fig. 4d) revealed a single class of high-affinity LH binding sites. The number of binding sites and receptor affinity did not change significantly from day 3, to days 10 and 14 (Table 3).

Competitive binding studies

The specificity and affinity of the equine LH receptor were established by binding competition studies. Highly purified hCG (CR-125) and ovine LH competed for binding of ^{125}I-labelled hLH at low doses (Fig. 5a; Table 4), as did commercial hCG (Chorulon) and PMSG (Folligon) preparations (Fig. 5b; Table 4). However, higher concentrations of eLH were required to compete for hLH binding. Moreover, eFSH had a similar potency to eLH, whereas microgram concentrations of ovine FSH were required to displace hLH binding (Fig. 5a). No significant competition for LH binding sites was observed with human or ovine prolactin, mouse EGF or the GnRH agonist buserelin (Fig. 5b).

LH binding to isolated luteal cells and tissue homogenates

The specific binding of ^{125}I-labelled hLH to mechanically dissociated and collagenase-dissociated equine luteal cells from tissue isolated on days 3, 10 and 14 of the luteal phase, and to homogenates of mare luteal tissue is shown (Fig. 6). After correction for DNA content, binding to luteal tissue homogenates and dissociated cells was not significantly different at the different stages of the luteal phase. Mechanically dissociated cells bound significantly more ($P < 0.05$) hLH than did collagenase-dissociated cells on days 10 and 14, but this difference was not significant on day 3.

Discussion

Histological examination of the mare corpus luteum at early, mid- and late dioestrus confirmed that the morphology of this tissue is unlike that of sheep and cattle. Whereas large and small cells are highly interspersed in ruminants, there are distinct trabeculae of small cells in the mare, with small cells lying between the large cells. Although Harrison (1946) suggested that the trabeculae contained cells that had originated from the theca interna, Van Niekerk *et al.* (1975) stated that only the granulosa layer contributed to the corpus luteum. Subsequent structural and functional studies have focused on the large cells in the mare corpus luteum (Levine *et al.*, 1979; Roser and Evans, 1983), and have not considered the contribution of small cells to luteal function.

Dissociation of luteal tissue from mares yielded both large and small cells. The proportion of large cells obtained by mechanical dissociation closely resembled that reported in intact tissue by Van Niekerk *et al.* (1975). The relatively higher proportion of small cells observed following collagenase treatment was probably due to a combination of the release of small cells from the matrix of the corpus luteum and the

Table 2. Progesterone secretion (ng per 10^5 cells h^{-1}) by cells obtained by mechanical and collagenase dissociation of equine (e) luteal tissue, and the effect of treatment with eLH (100 ng ml^{-1}) and hCG (100 iu ml^{-1}) (mean ± SEM)

	Day 3 of dioestrus			Day 10 of dioestrus			Day 14 of dioestrus		
Procedure	Basal	+ eLH	+ hCG	Basal	+ eLH	+ hCG	Basal	+ eLH	+ hCG
Mechanical	54 ± 7	45 ± 14	34 ± 11	1264 ± 272	1516 ± 851	1205 ± 623	720 ± 118	824 ± 305	338 ± 177
Collagenase	224 ± 52	203 ± 114	200 ± 122	865 ± 181	528 ± 302	367 ± 210	3054 ± 558	3685 ± 1615	2894 ± 1061

n = at least four experiments for each stage of the luteal phase.
Both mechanically dispersed and collagenase-dispersed cells on days 10 and 14 secreted significantly more progesterone than did those on day 3 ($P < 0.01$).
Collagenase-dispersed cells on days 3 and 14 secreted more progesterone than did mechanically dispersed cells from these days ($P < 0.01$).
On day 10 secretion of progesterone by mechanically dispersed and collagenase-dispersed cells was not significantly different ($P > 0.1$).
Addition of eLH (10, 50, 500 ng ml^{-1}) or hCG (0.1, 10 iu ml^{-1}) did not significantly increase progesterone secretion (data not shown).

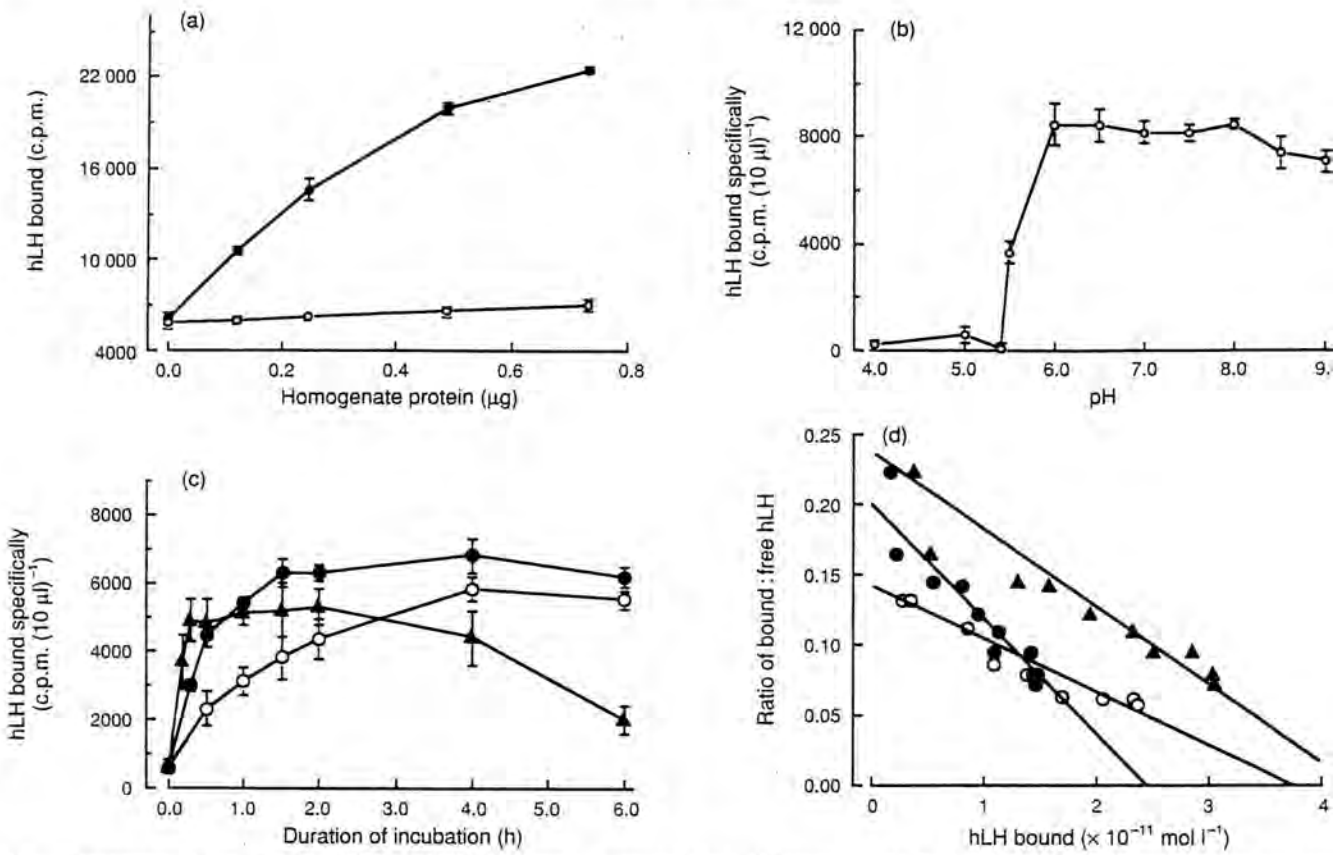

Fig. 4. Properties of the binding of ^{125}I-labelled hLH tracer to equine luteal LH receptors. (a) Binding of hLH tracer to increasing concentrations of mare corpora lutea homogenate in the absence (●) or presence (○) of 50 iu unlabelled hCG. (b) Specific binding of hLH tracer to the corpora lutea homogenate in media at different pH values. (c) Specific binding of hLH tracer to mare corpora lutea homogenate following incubation for various times at either 4°C (○), 20°C (●) or 37°C (▲). (d) Scatchard analyses of ^{125}I-labelled hLH binding to equine luteal tissue homogenates 3 (○), 10 (●) and 14 (▲) days after ovulation. Points represent means ± SEM for 1–5 separate experiments performed in triplicate.

preferential destruction of large cells. Cell sizes reported here are in agreement with previously published sizes for dispersed cells (Watson and Sertich, 1990) and cells in intact tissue (Van Niekerk *et al.*, 1975). Staining for 3β-HSD was localized mainly to the large cells, with virtually no staining of small cells from either mechanically or collagenase-dispersed tissue; this result concurs with the localization of 3β-HSD in granulosa cells of the preovulatory follicles of mares (Hay *et al.*, 1975). However, it is possible that the concentrations of 3β-HSD in the small cells in our preparations were too low to be detected in this assay. In contrast to our results, Kelly *et al.* (1988) reported two luteal cell populations, both of which were smaller than 25 μm in diameter, with the majority of 3β-HSD staining localized in a cell population measuring 7–18 μm in diameter (although only 30% of the small cells stained for 3β-HSD in this study). However, these cells were obtained after up to 20 h of collagenase digestion, and it is likely that the fragile large luteal cells had been destroyed.

Despite the lack of obvious staining for 3β-HSD in the small cells, our results showed that both mechanically and collagenase-dispersed cells secreted progesterone. Furthermore, one of our equine luteal cell preparations, which happened to

Table 3. Number of LH binding sites and affinity constants for equine luteal tissue 3, 10 and 14 days after ovulation

Day	B_{max} ($\times 10^{-11}$ mol l^{-1} μg^{-1} DNA)	K_a ($\times 10^{10}$ l mol^{-1}
3	4.30	0.382
3	2.25	1.179
3	3.73	0.556
Mean	3.43 ± 0.61	0.71 ± 0.24
10	11.11	0.254
10	8.16	0.764
10	2.32	0.637
10	7.22	0.315
10	9.83	0.229
10	3.59	0.152
Mean	7.04 ± 1.40	0.39 ± 0.10
14	4.75	0.841

B_{max} and K_a on days 3 and 10 were not significantly different ($P > 0.1$).

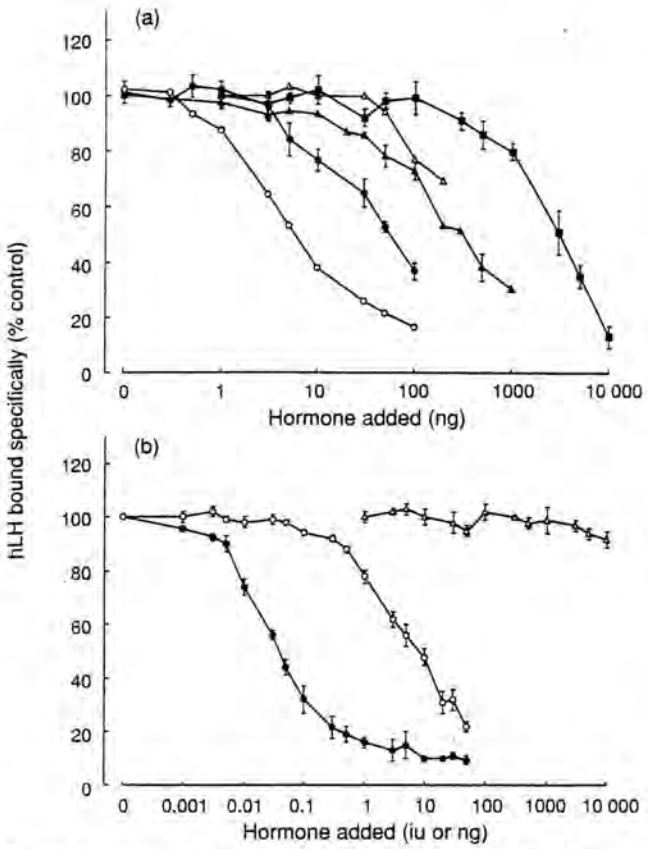

Fig. 5. Specificity of equine luteal LH binding sites. Displacement of ^{125}I-labelled hLH with increasing concentrations of (a) hCG (CR125; ○), oLH (S-23; •), eLH (E263B; ▲), eFSH (E276B; △) and oFSH (S-14; ■) or (b) Folligon (PMSG; ○) (iu), Chorulon (hCG; •) (iu) and mEGF, oPRL, hPRL and the GnRH agonist buserelin (△) (ng). EGF: epidermal growth factor; PRL: prolactin.

Table 4. Concentrations required for half-maximal displacement of ^{125}I-labelled hLH binding (IC_{50}) to equine luteal tissue homogenates by exogenous hormones

Hormone	*n*	IC_{50} (ng or iu)
hCG (CR-125)	2	8 ng
oLH (S-23)	2	60 ng
eLH (E236B)	3	350 ng
eFSH (E276B)	1	350 ng
oFSH (S-14)	1	2000 ng
Chorulon (hCG)	5	0.05 iu
Folligon (PMSG)	4	9.0 iu

PMSG: pregnant mares' serum gonadotrophin.
Values are means of 1–5 separate displacement experiments performed in triplicate.

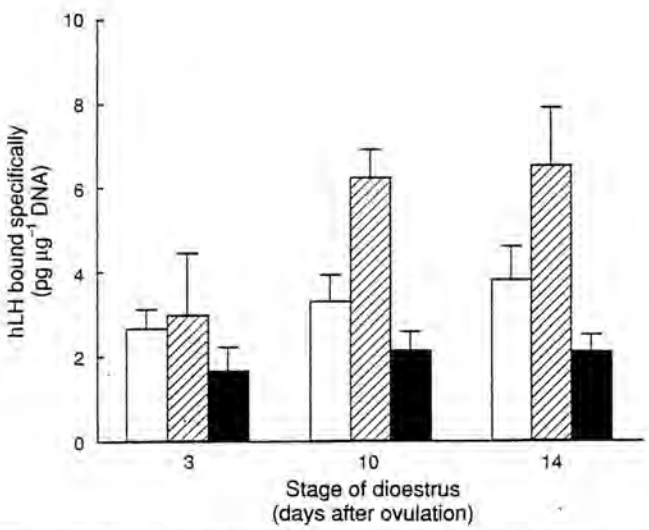

Fig. 6. LH binding to equine luteal tissue homogenate (□) and to mechanically dissociated (▨) and collagenase-dissociated (■) luteal cells at different stages of dioestrus. The average binding corresponds to tissue and cells from at least four animals, and binding was performed in triplicate on each sample. Bars represent SEM. LH binding to luteal tissue homogenates on days 3, 10 and 14 of dioestrus was not significantly different ($P > 0.1$). Mechanically dissociated cells bound significantly more ($P < 0.05$) hLH than did collagenase-dissociated cells on days 10 and 14.

consist entirely of small cells, secreted a high concentration of progesterone (2007 ng per 10^5 cells h^{-1}), indicating that at least some of the small cells derived from the corpus luteum of mares are steroidogenic.

Mechanically dispersed and collagenase-dissociated luteal cell preparations failed to respond to exogenous gonadotrophin with increased progesterone secretion. A lack of response to exogenous gonadotrophin has been reported for sheep, cattle and mare luteal cells cultured in serum-containing media (Pate and Condon, 1982; Hoyer *et al.*, 1988; Watson and Sertich, 1990), suggesting that the cells may have been maximally stimulated by LH present in the serum (Watson and Sertich, 1990). Indeed, stimulation of progesterone secretion by LH has been reported previously for equine (Kelly *et al.*, 1988) and rat luteal cells (Nelson *et al.*, 1992) cultured in serum-free medium. The presence of fetal calf serum in the dissociation medium of collagenase-dispersed cells in the present study could explain the lack of stimulation by LH, as these cells may have been maximally stimulated by this treatment. However, this does not explain why mechanically dispersed cells, which had not been exposed to fetal calf serum, were also unstimulated. Furthermore, human luteal cells isolated mechanically in media without fetal calf serum are capable of responding to both hCG and dibutyryl cAMP (T. A. Bramley, unpublished).

Lack of responsiveness to LH did not appear to be due to a lack of LH receptors on mare luteal cells. Our LH binding data agree in general with those of Roser and Evans (1983), which showed a single population of high-affinity LH receptors on equine luteal tissue. However, there were a number of important differences between our data and those of other studies. We found that the binding of LH to luteal tissue and dissociated cells was not different on days 3, 10 and 14 after ovulation. In contrast, Roser and Evans (1983) described a 21-fold increase in unoccupied receptors in equine corpus luteum between day 1 and day 14 of the cycle, and studies in other species have generally described maximal LH binding in the mid-luteal

phase (pigs: Ziecik *et al.*, 1980; sheep: Diekman *et al.*, 1978; cattle: Spicer *et al.*, 1981; humans: Bramley *et al.*, 1987). However, the calculations of Roser and Evans were normalized to the mass of the corpus luteum, which may have underestimated the number of LH binding sites in the early corpus luteum because of the large amount of fibrin and red blood cells present in the tissue at this stage (Pierson and Ginther, 1985). We have shown a higher degree of LH binding than that reported by Roser and Evans (1983) and Stewart and Allen (1979), although it is difficult to compare studies, particularly that of Roser and Evans (1983), as the units in which binding was expressed were not the same. Furthermore, the number of LH binding sites may have been significantly underestimated by other groups, since separation of bound from free hormone by centrifugation alone fails to recover all the LH-receptor–hormone complexes present (Bramley, 1981).

Roser and Evans (1983) reported a fivefold increase in receptor affinity between day 1 and day 13, and Ziecik *et al.* (1980) showed that the affinity constant of the porcine luteal LH receptor increased significantly between day 10 and day 16. However, most other studies have failed to demonstrate a change in LH receptor affinity in corpora lutea of other species (cattle and sheep: Diekman *et al.*, 1978; Rao, 1979; humans: Bramley *et al.*, 1987), and we observed no significant change in receptor affinity from day 3, to days 10 and 14 (although binding affinity did vary markedly in different corpora lutea at the same stage of the luteal phase).

Cells obtained by mechanical dissociation of luteal tissue appeared to bind more LH than did homogenates of luteal tissue, although the difference was not significant. Mechanical dissociation of luteal tissue yielded a high proportion of large luteal cells, whereas tissue homogenates contain many nonluteal cells in addition to LH-binding luteal cells. Hence, expression of the number of LH receptors on a per cell (DNA) basis could account for the difference in binding between cells obtained by mechanical dissociation and tissue homogenates. Similarly, a significantly lower degree of LH binding in cells obtained by collagenase dissociation of luteal tissue on days 10 and 14 compared with mechanical dispersion may reflect the presence of other nonluteal cell types within the corpus luteum (endothelial cells and fibroblasts) that do not bind hormone but that survive cell dissociation by collagenase and increase the DNA content of the cell pellet. Since the two dissociation methods yield quite different cell populations, it is not clear at present whether mechanically dissociated cells possess more LH receptors, or whether collagenase treatment has resulted in some receptor loss or damage.

The results obtained in this study are particularly interesting for several reasons. First, large luteal cells isolated from ovine and bovine corpora lutea possess few or no LH receptors (Fitz *et al.*, 1982; Hoyer and Niswender, 1985). However, in our study, the binding of LH to cells obtained by mechanical dissociation (with many large cells) was greater than that to cells obtained by collagenase dissociation (predominantly small cells). This suggests that large cells from the mare corpus luteum bind LH.

Second, damage to LH binding sites during collagenase dispersion of equine luteal cells cannot fully explain the lack of effect of exogenous LH on progesterone secretion, as cells obtained by mechanical dissociation were also unresponsive to LH, and both cell preparations bound LH. Rather, these results suggest that luteal tissue from mares is not responsive to LH at the stages examined in this study or that hormonal control of the mare corpus luteum may differ from that in other species.

Third, although cells derived from the theca interna develop into small luteal cells of the corpus luteum in other species, it is believed that only the granulosa cells of the mare preovulatory follicle contribute to the fully formed corpus luteum (Van Niekerk *et al.*, 1975). However, we have observed LH binding and progesterone secretion by collagenase-dissociated luteal tissue that contained a high proportion of small cells, suggesting that at least a proportion of small cells of the mare corpus luteum may be luteal in nature.

To our knowledge, this is the first report to compare cell populations obtained by mechanical and collagenase dissociation of luteal tissue with regard to morphology, progesterone secretion and LH binding. Most functional studies of cells from the corpus luteum have used enzymatic dissociation or a combination of methods using physical disruption of the tissue followed by enzyme treatment to increase cell yield. Although not all commercially available collagenase preparations would necessarily have the same effect, we have shown that enzymatic dissociation of the corpus luteum may provide cell populations that are unrepresentative of this tissue.

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Particulate Progesterone-Binding Sites in the Equine Corpus Luteum[1]

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ABSTRACT

We have subjected homogenates of mid-luteal phase equine CL to fractionation on continuous sucrose density gradients and have assayed gradient fractions for progesterone content, for specific binding of [^{3}H]progesterone and [^{125}I]-labeled LH, and for a number of marker activities for the major intracellular organelles.

Progesterone content (a reflection of progesterone synthesis) equilibrated in membrane fractions with a buoyant density of 20–24% sucrose (1.08–1.10 g/cm^3). In contrast, specific binding of [^{3}H]-labeled progesterone equilibrated at a buoyant density of 25–34% sucrose (1.10–1.14 g/cm^3), similar to that of markers for luteal cell surface membranes (LH receptor, alkaline phosphatase) but distinct from that of other major luteal cell organelles. Binding of radiolabeled progesterone to dextran-charcoal-stripped equine luteal membrane fractions pooled from the 20–24% sucrose (1.08–1.10 g/cm^3) region of the gradients was low, but it could be stimulated markedly and in a dose-dependent fashion by digitonin. Other detergents and cardiotonic steroids had no effect. In the presence of digitonin, [^{3}H]progesterone binding increased with increasing membrane concentration, and binding was dependent on the pH, duration, and temperature of incubation. Unlabeled progesterone competed for the binding of [^{3}H]progesterone with a concentration required to reduce specific binding by 50% (IC_{50}) of 70 nM, whereas 17-hydroxyprogesterone, dihydrotestosterone, and testosterone had lower potencies (IC_{50}, 900–3130 nM). Dehydroepiandrosterone (14 μM), pregnenolone, and androstenedione were even less effective (250 μM); and millimolar concentrations of estrogen, steroid conjugates, corticosteroids, cholesterol, inhibitors of luteal steroidogenic enzymes, and the progesterone receptor antagonist RU486 failed to compete. These results indicate the presence of specific, high-affinity binding sites for progesterone associated with luteal cell-surface membranes of the mare.

INTRODUCTION

The CL is essential for the maintenance of the first half of pregnancy in the mare by secretion of the steroid hormone, progesterone. Despite the central role of this hormone in reproduction, little is known of the mechanism of progesterone secretion in the mare. Originally, it was believed that steroids left the cell by passive diffusion [Enders, 1973]. Later studies in ruminants implicated electron-dense granules secreting progesterone by exocytosis [Gemmell & Stacy, 1979; Sawyer et al., 1979]. However, these granules were shown subsequently to contain oxytocin and neurophysin, which differed from progesterone in their release characteristics [Sernia et al., 1982; Hirst et al., 1986].

Recent work has identified a unique particulate subcellular fraction within the CL of a number of species, including sows [Bramley & Menzies, 1988a; Menzies & Bramley, 1994], cattle [Rae et al., 1992], sheep [Bramley & Menzies, 1994], humans [Bramley & Menzies, 1988b], and goats (Mani, Menzies, Bramley, & Watson, unpublished observations), that can specifically bind radiolabeled progesterone with high affinity. In isolated luteal membranes, these sites appear to be occupied by endogenous steroid that cannot be removed by stripping with dextran-charcoal; however, inclusion of digitonin in the binding assay in vitro stimulates [^{3}H]progesterone binding, apparently by displacing endogenous steroid(s) and unmasking the high-affinity progesterone-binding sites (Rae and Bramley, unpublished data).

Steroidogenesis and control of luteal function in the mare may differ from that in other species [Short, 1964; Channing, 1969; Condon et al., 1979; Watson & Sertich, 1990]. Knowledge of the mechanism of release of progesterone by the mare CL should give a better understanding of the control of luteal function and lead to new methods for the regulation of fertility in equids. In the present study, we employed density gradient fractionation with markers for various intracellular organelles 1)

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to determine whether or not the mare CL has a specific steroid-binding fraction and 2) to determine the subcellular component with which progesterone binding was associated.

MATERIALS AND METHODS

Materials

All chemicals, inhibitors, and reagents were of analytical grade and were obtained from either Sigma Chemical Co. (Poole, Dorset, UK) or from BDH (Poole, Dorset, UK). Radiolabeled [1,2,6,7-^{3}H]progesterone (100 Ci/mmol) was purchased from Amersham International plc, Amersham (Bucks, UK), and [^{125}I]-labeled human LH (hLH) was purchased from Dr. Keith Ferguson, Chelsea Hospital for Women, London, UK. Human CG (Chorulon; 1500 IU hCG/ampule; for measurement of nonspecific [^{125}I]-LH binding) was purchased from Intervet Laboratories (Cambridge, UK).

Methods

CL were recovered postmortem from four mares in the mid-luteal phase of the cycle. Each individual CL was weighed, trimmed free of stroma and connective tissue, and homogenized in a Polytron homogenizer in ice-cold SET buffer (0.3 mol/L sucrose, 10 mmol/L Tris, 1 mmol/L EDTA buffer, pH 7.4; 100 mg wet weight/ml) with two 10-sec bursts at full speed separated by a 1-min cooling period in ice. After filtration through cheesecloth, aliquots (3 ml) of each homogenate were layered over 30 ml continuous sucrose density gradients (10–50%; 1.03–1.23 g/cm^3) prepared by the method of Stone [1974] and centrifuged at 30,000 $\times$ $_g$ for 2 h (4°C) in a vertical tube rotor (Sorvall, Du Pont, UK). After centrifugation, tubes were placed in ice, and thirty 1-ml (36 drop) fractions of each gradient were collected through use of a Buchler-Searle Autodensiflow (Fort Lee, NJ) fractionator equipped with a meniscus-sensitive probe. The hollow, meniscus-tracking probe was coupled to a peristaltic pump and fraction collector, enabling fractionation of sucrose gradients from the top to the bottom of the tube in a highly reproducible manner.

Assays

The sucrose concentration of each fraction was measured by refractive index (Abbe refractometer, Otago, Japan), and buoyant density was calculated from standard tables.

Specific binding of [^{125}I]-hLH was measured by the method of Broadley et al. [1994]. Luteal cell-surface membrane and intracellular organelle markers were measured in triplicate by minor adaptations of previously published methods [Bramley & Menzies, 1988a], optimized for the equine CL. Buoyant density of membranes and organelles was calculated as described previously [Bramley & Menzies, 1988a].

The methods used for the measurement of progesterone content of density gradient fractions by specific RIA and for the specific binding of [^{3}H]progesterone have been described in detail previously [Bramley & Menzies, 1994]. Fractions with high activity were pooled and were treated twice with an equal volume of ice-cold dextran-coated charcoal (DCC; 1.25 g activated charcoal, 0.125 g dextran T70 in 100 ml of 40 mmol/L Tris-HCl buffer, pH 7.4) followed by centrifugation at 2500 $\times$ g for 10 min to remove endogenous unbound steroids.

RESULTS

Markers for the major luteal cell organelles were found to equilibrate at different buoyant densities: plasma membrane markers (alkaline phosphatase, Fig. 1a; LH receptor, Fig. 1b) equilibrated at 25-33% sucrose (1.10–1.14 g/cm^3); cytosol markers, at the top of the gradient; nuclei, at $>$ 1.20 g/cm^3; and lysosomes, at 43% sucrose (1.17–1.19 g/cm^3; Fig. 1b). Mitochondria (cytochrome oxidase; Fig. 1c) equilibrated at 33–38% sucrose (1.14–1.17 g/cm^3). Most of the endogenous progesterone content of the equine luteal homogenate was recovered in gradient fractions with a buoyant density of 19–24% sucrose (1.08–1.10 g/cm^3), with little progesterone recovered in cytosolic or nuclear fractions (Fig. 1c).

Despite charcoal stripping to remove endogenous unbound steroids, fractions pooled from regions of the gradients enriched in progesterone content (1.08–1.10 g/cm^3) bound little [^{3}H]progesterone in vitro, even at high membrane concentrations (Fig. 2a). However, in the presence of digitonin, progesterone binding increased markedly with increasing membrane concentration (Fig. 2a). Digitonin alone had little effect on progesterone binding, but stimulated binding in the presence of luteal membranes in a dose-dependent fashion, reaching a plateau at about 250 μmol/tube (Fig. 2b).

There was no specific binding of [^{3}H]progesterone to luteal membrane gradient fractions in the absence of digitonin, but a broad peak of binding equilibrating at a buoyant density of

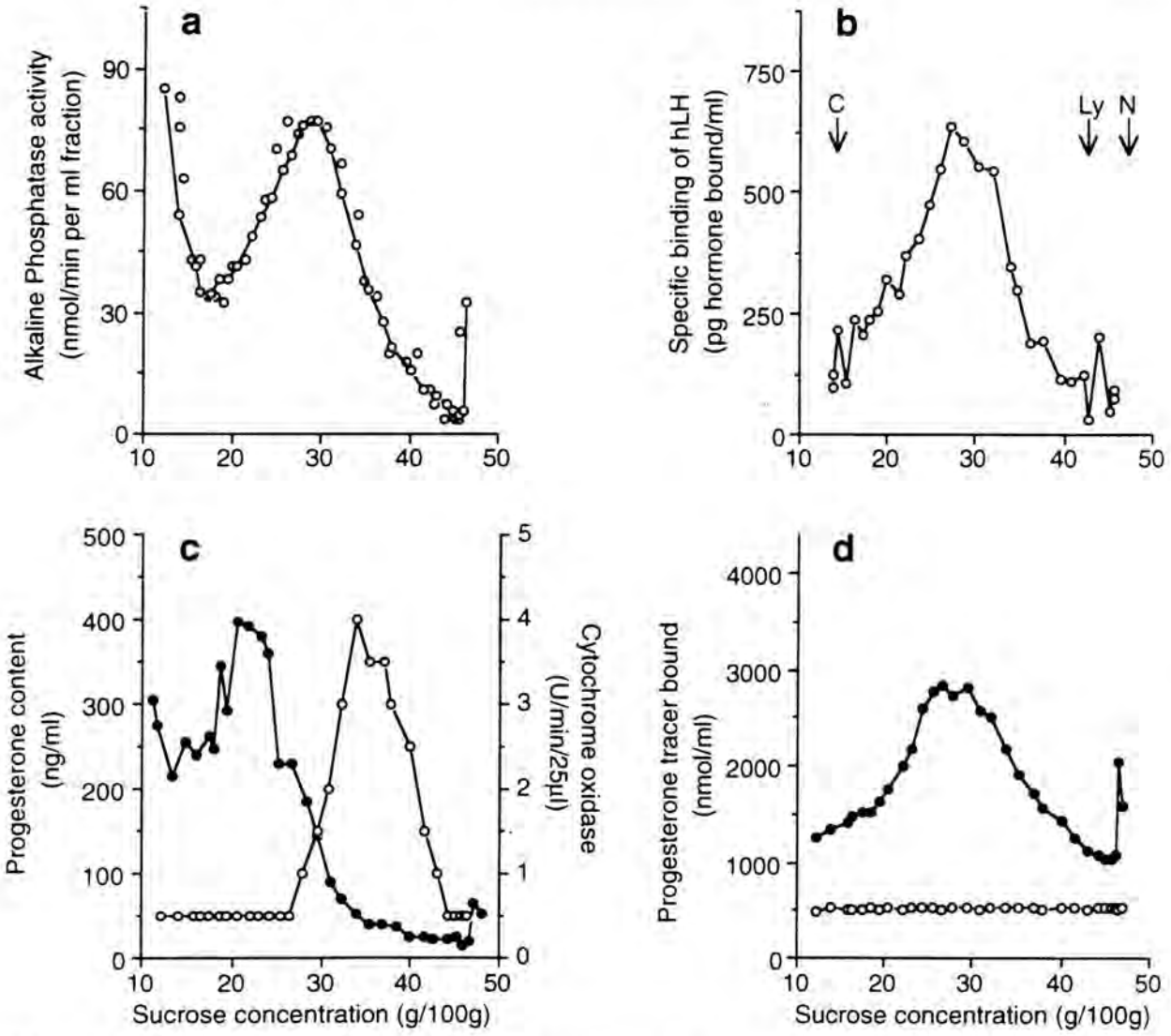

FIG. 1. Subcellular fractionation of equine luteal homogenates on continuous sucrose density gradients. Aliquots (3 ml) of homogenates of mid-luteal equine CL were applied to 30 ml continuous (10–50 g/cm^3) sucrose gradients and spun at 30,000 $_{gav}$ for 2 h (4°C). Fractions (1 ml) were collected and assayed for (a) alkaline phosphatase activity; (b) specific binding of [^{125}I]-labeled hLH; (c) progesterone content by RIA (solid circles) and cytochrome oxidase activity (open circles); (d) specific binding of [^{3}H]progesterone in the absence (open circles) or in the presence of 400 µmol/tube of digitonin (solid circles). Figures depict data from a single representative experiment. Similar profiles were obtained in separate fractionation experiments with mid-luteal tissue from the other three mare CL tested. Arrows in Figure 1b indicate the buoyant density peaks of markers for cytosol (C; glucose 6-phosphate dehydrogenase), lysosomes (Ly; *N*-acetyl-β-glucosaminidase), and nuclei (N; DNA content).

1.10–1.14 g/cm^3 was observed when in vitro binding assays were conducted in the presence of digitonin (400 µmol/tube; Fig. 1d).

The effects of digitonin on progesterone binding were specific; and a number of cationic, anionic, and nonionic detergents failed to mimic the effects of digitonin by increasing [^{3}H]progesterone binding. Furthermore, other cholesterol-binding saponins (filipin, nystatin, tomatine) and cardiotonic steroids (ouabain, digoxin, digitoxin, digoxigenin) failed either to stimulate [^{3}H]progesterone binding to equine luteal membranes in the absence of digitonin or to inhibit [^{3}H]progesterone binding in the presence of digitonin (data not shown).

Binding of [^{3}H]progesterone to equine luteal membranes was dependent on the pH (pK, 6.0), temperature, and duration of incubation (data not shown). Moreover, equine luteal membrane binding sites were specific for progesterone. The specific binding of [^{3}H]progesterone was inhibited by low doses of unlabeled progesterone (IC_{50}, 70 nM), but other steroids were much less efficacious (Fig. 3; Table 1). In addition, other radiolabeled steroids were not bound appreciably by this fraction, even in the

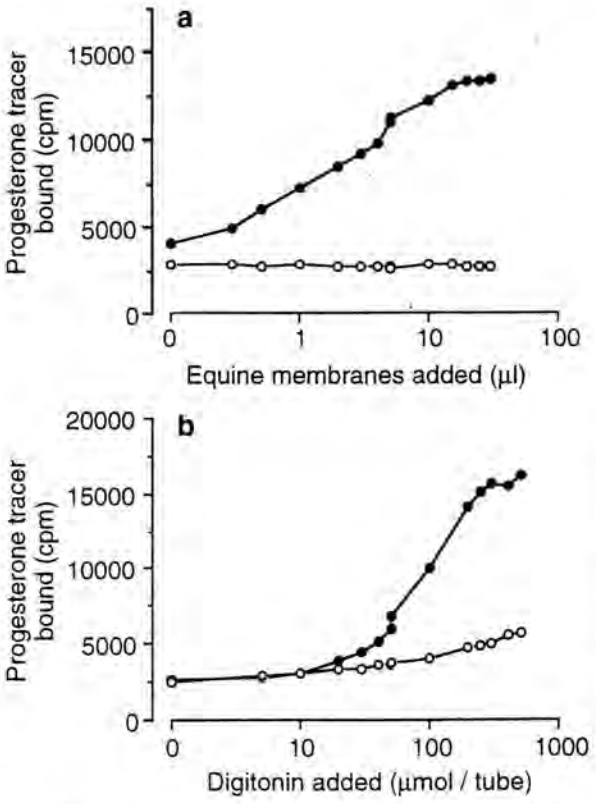

FIG. 2. Effects of digitonin on [^{3}H]progesterone binding to equine luteal membranes in vitro. a) Effects of increasing equine luteal membrane concentration (20–24% sucrose; 1.08–1.10 g/cm^3) in the presence (solid circles) or the absence (open circles) of digitonin (400 µmol/tube). b) Effects of increasing digitonin concentration in the presence (solid circles) or the absence (open circles) of equine luteal membranes (10 µl). Incubation was for 2 h at 4°C. Points are means of triplicate determinations. SEM values were generally smaller than the point size. Similar data were obtained in two similar, but less complete, experiments.

TABLE 1. Specificity of equine luteal progesterone-binding sites.*

Substance tested	IC_{50} (nM)
Progesterone	70 ± 12 (4)
17α-Hydroxyprogesterone	914 ± 99 (3)
Dihydrotestosterone	1250 ± 138 (2)
Testosterone	3130 ± 360 (2)
Dehydroepiandrosterone	14,200 ± 5800 (2)
Pregnenolone	245,000 ± 44,500 (3)
Androstenedione	250,000 ± 50,000 (2)
Estradiol	>1,000,000 (2)
RU486	>1,000,000 (2)

*Values are means ± SEM for (n) determinations.

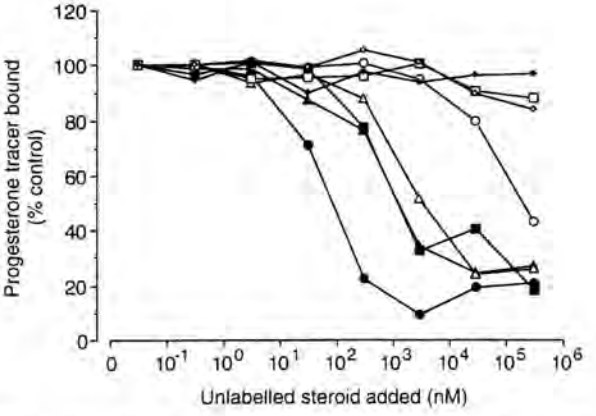

FIG. 3. Hormonal specificity of equine luteal [^{3}H]progesterone-binding sites. Aliquots (10 µl) of dextran-charcoal-stripped equine luteal membrane fractions (25–33% sucrose; 1.10–1.14 g/cm^3) were incubated for 2 h at 4°C with [^{3}H]progesterone tracer (50,000 cpm/tube) and digitonin (400 µmol/tube) in the absence or in the presence of increasing concentrations of unlabeled steroids. Solid circles, progesterone; open circles, pregnenolone; open triangles, testosterone; solid triangles, 17-hydroxyprogesterone; open squares, cortisol; solid squares, dihydrotestosterone; open diamonds, cholesterol; solid diamonds, RU486. Points are means for one to four separate experiments in triplicate on four mare CL membrane fractions. SEM values are omitted for clarity. Similar results were obtained in other less complete experiments (data not included).

presence of digitonin, with the exception of [^{3}H]pregnenolone; however, although [^{3}H]pregnenolone tracer bound, unlabeled progesterone competed for radiolabeled pregnenolone binding at much lower concentrations than did unlabeled pregnenolone (data not shown).

DISCUSSION

We have described herein the presence of specific, high-affinity (IC_{50}, 70 nM) particulate binding sites for progesterone in the equine CL. Endogenous progesterone equilibrated at a density of 1.08–1.10 g/cm^3, a buoyant density similar to that of luteal endoplasmic reticulum (ER) membranes in other species [Bramley & Menzies, 1988a,b, 1994; Menzies & Bramley, 1994]. Little progesterone was associated with fractions enriched in either cytosol or nuclei (Fig. 1c), in contrast to the usual subcellular localization of classical progesterone receptors. The enzymes involved in the synthesis of progesterone are thought to be associated with the smooth ER, suggesting that the 1.08–1.10 g/

cm^3 fraction may represent newly synthesized progesterone associated with the smooth ER (Fig. 1c). In contrast, in the presence of digitonin, [^{3}H]progesterone tracer was bound in vitro to fractions with a buoyant density of 1.10–1.14 g/cm^3 (Fig. 1d), a buoyant density similar to that of luteal cell-surface membrane markers (Fig. 1, a and b) but distinct from the profile of markers for cytosol, nuclei, mitochondria, and lysosomes (Fig. 1, b and c). This finding differed from those from fractionation studies of ovine and porcine CL in which both endogenous and exogenous progesterone content and [^{3}H]progesterone binding measured in vitro in the presence of digitonin equilibrated at a buoyant density coinciding with that of markers for smooth ER membranes [Bramley & Menzies, 1994; Menzies & Bramley, 1994]. This suggests that the subcellular localization of steroid-binding sites in the mare CL may differ in this regard from that in other species.

The demonstration of specific progesterone binding to equine luteal membranes in vitro required the inclusion of digitonin in the binding assay. Digitonin is a steroidal detergent (saponin) and may stimulate binding by disrupting resealed membrane vesicles, allowing access of tracer to previously inaccessible progesterone-binding sites on the internal surface of the vesicle. However, a range of detergents that effectively abolish membrane permeability barriers to the passage of impermeant molecules in other systems failed to stimulate progesterone binding. Digitonin also has structural similarities to digoxin and other cardiotonic steroids; however, a range of cardenolide steroids had neither stimulatory nor inhibitory effects on progesterone binding to equine membranes (Table 1), suggesting that the action of digitonin was not attributable to its cardiotonic activity.

Digitonin forms one-to-one complexes with steroids with the 3β-hydroxy Δ^4 configuration possessing a side chain at C17 [Severs & Robenek, 1983]; it may therefore act by binding to cholesterol or another endogenous luteal steroid. Indeed, experiments with bovine luteal membranes have indicated that digitonin precomplexed to cholesterol or pregnenolone no longer stimulated [^{3}H]progesterone binding to bovine CL membranes, suggesting that the ability to complex with an endogenous steroid may be important in the mechanism of action of digitonin (Rae and Bramley, unpublished data). However, this action would seem to be specific, since other cholesterol-active compounds (filipin, nystatin, and tomatine [Severs & Robenek, 1983]) failed to reproduce the effects of digitonin on binding.

The simplest explanation of our data is that equine luteal steroid-binding sites are already occupied by an endogenous, tightly bound (luteal membrane?) steroid(s) that is not removed by stripping with dextran-charcoal. We suggest that digitonin interacts specifically with this endogenous steroid(s) to form a digitonide-steroid complex; this removes digitonin from the binding site and enables binding of exogenous [^{3}H]progesterone to the vacant binding site. Experiments are in progress to verify this explanation and to identify the endogenous digitonin-active steroid(s).

Our results have indicated the presence of a new class of high-affinity, progesterone-specific binding sites in the equine CL that appear to be associated with the luteal cell-surface membrane. These sites may be involved in the mechanism of progesterone secretion from the cell. However, the observation that endogenous progesterone content (Fig. 1c) and [^{3}H]progesterone binding in vitro (Fig. 1d) could be resolved in these gradients suggests that these sites are probably not involved in steroid packaging for secretion in the mare CL.

Alternatively, these specific progesterone-binding sites may be involved in mediating autocrine/paracrine effects of progesterone on luteal cell function. "Classical" progesterone receptors have been described in luteal tissue from a number of species [Press & Green, 1988; Korte & Isola, 1988; Isola et al., 1987; Hild-Petito et al., 1988; Iwai et al., 1990; Horie et al., 1992] and may be involved in local regulation of luteal cell function. However, the inability of the classical progesterone receptor antagonist, RU486, to compete for particulate luteal progesterone-binding sites (Table 1) clearly distinguished the particulate steroid-binding sites from classical progesterone receptors. Hence, RU486 will be a useful tool with which to distinguish those actions of progesterone on the CL that are mediated by classical progesterone receptors from those mediated by cell-surface progesterone-binding sites.

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Detection of steroidogenic acute regulatory protein in equine ovaries

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A steroidogenic acute regulatory (StAR) protein has been identified in several species as a probable important rate-limiting step in steroidogenesis. This protein is believed to be responsible for transporting cholesterol from the outer to the inner mitochondrial membrane. It is known that equine chorionic gonadotrophin (eCG) stimulates steroidogenesis in the corpora lutea of early pregnant mares and that eCG also upregulates StAR mRNA in bovine ovaries. In the present study, ovarian tissue from cyclic and early pregnant mares was immunostained to detect the distribution of the StAR protein. Western blot analysis was performed, followed by phosphor imaging to establish whether the onset of eCG secretion in pregnancy was associated with increased expression of the StAR protein. Immunostaining for StAR was confined to the theca interna of growing and preovulatory follicles, but 24 h after treatment with hCG, some granulosa cells were positively stained. Positive staining was confined to the large luteal cells of the equine corpus luteum. There was no difference in the distribution of immunostaining before or after onset of eCG secretion in pregnant mares, but increased amounts of StAR were detected in corpora lutea from mares at day 40 or day 41 of pregnancy compared with non-pregnant mares and mares at days 20–30 of pregnancy.

Introduction

The first enzymatic step in the biosynthesis of all steroid hormones is the cleavage of the side chain of cholesterol to form the first steroid produced in all steroidogenic tissues, pregnenolone. Cytochrome P450 side chain cleavage enzyme (P450scc), which catalyses synthesis of pregnenolone, has long been considered to be the rate-limiting enzyme in steroid biosynthesis (Miller, 1988). However, subsequent *in vitro* work showed that this was not the case as the activity of P450scc was largely unaffected by trophic hormone stimulation (Lambeth *et al.*, 1982; Tuckey, 1992). Many years ago it was suggested that a highly labile protein was required for the acute production of steroids in response to hormone stimulation (Garren, 1968). Later work localized this regulated step to the delivery of cholesterol, the substrate of pregnenolone, to the inner mitochondrial membrane (Cooke *et al.*, 1975; Crivello and Jefcoate, 1978). A steroidogenic acute regulatory protein (StAR) has now been identified and characterized and is believed to be responsible for transporting cholesterol from the outer to the inner mitochondrial membrane, possibly the first rate-limiting step in steroidogenesis.

The StAR protein sequence is highly conserved and has greater than 90% similarity among species studied to date (Stocco and Clark, 1996). Most studies have investigated mRNA expression for StAR and this seems to be closely related to StAR protein concentrations, at least in the bovine corpus luteum (Pescador *et al.*, 1996). Tissue specificity for StAR appears to vary among species. In the bovine Graafian follicle, StAR appears to be present exclusively in the theca interna (Soumano and Price, 1997; Bao *et al.*, 1998), although an earlier study reported its presence in both granulosa and theca cells (Pescador *et al.*, 1996). StAR mRNA is readily detected in granulosa cells collected from women after superovulation (Sugawara *et al.*, 1995) and it has been suggested that StAR expression is induced by gonadotrophin treatment. In cattle undergoing ovarian hyperstimulation with eCG, the amount of thecal StAR mRNA increased markedly compared with cows treated with FSH (Soumano and Price, 1997), and therefore this effect appears to be related to the LH activity of the treatment.

In equine preovulatory follicles, StAR mRNA was undetectable in granulosa cells before treatment with hCG, but expression increased markedly 30 h after hCG treatment. In contrast, increased amounts of StAR mRNA were present initially in theca interna and these decreased markedly after hCG treatment (Kerban *et al.*, 1999). StAR mRNA is present in the corpora lutea of other species (Hartung *et al.*, 1995; Juengel *et al.*, 1995; Sugawara *et al.*, 1995; Pescador *et al.*, 1996). Granulosa cells are the predominant types of cell in the equine corpus luteum (Harrison, 1946; Van Niekerk *et al.*, 1975) and therefore it might be predicted that large luteal cells in the equine corpus luteum would express StAR mRNA and protein. In the pregnant mare, the onset of eCG secretion at about day 35 coincides with an increase in luteal steroidogenesis and a relative shift towards androgen and oestrogen synthesis (Daels *et al.*, 1991, 1996). This increase in

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steroidogenesis is induced by eCG (Daels *et al.*, 1998) and is associated with an upregulation of both the mRNA encoding the enzyme $P450_{C17}$ (Albrecht *et al.*, 1997) and the protein itself (Albrecht and Daels, 1997; Rodger *et al.*, 1998). There is currently no information on StAR expression in the equine corpus luteum and it is possible that the onset of eCG secretion in pregnancy upregulates StAR mRNA, as occurs in cyclic cattle treated with eCG (Soumano and Price, 1997). Furthermore, in several experimental systems a direct relationship has been demonstrated between StAR expression and steroid synthesis (Juengel *et al.*, 1995; Pescador *et al.*, 1996; Townson *et al.*, 1996). In the present study, follicles and corpora lutea were collected from cyclic and early pregnant mares before and after the onset of eCG secretion and the tissues were examined by immunohistochemistry and western blotting techniques to establish whether the onset of eCG secretion in pregnancy is associated with a redistribution of StAR expression or an alteration in the level of StAR expression.

Materials and Methods

Ovaries were obtained from 18 mares by hemiovariectomy or after the animals were killed. The study was performed with the approval of the Home Office under the *Animals (Scientific Procedures) Act 1986*. Hemiovariectomies were performed via a vaginal incision after induction of appropriate analgesia and sedation (Watson and Sertich, 1990). Local anaesthetic was applied to the broad ligament before application of a chain ecraseur. After surgery, mares were given broad-spectrum antibiotic cover and analgesia was administered as necessary. The mares were aged between 4 and 20 years and weighed 250–380 kg. Tissue from five preovulatory follicles, two of which were collected 24 h after i.v. administration of 2500 iu hCG (Chorulon; Intervet Laboratories, Cambridge), five small follicles (1–25 mm), five mid-luteal corpora lutea from non-pregnant mares, and corpora lutea from two mares at day 20 of pregnancy and three mares at day 40–42 of pregnancy were embedded in OCT compound (Miles Inc, Elkhart, IN) on cork discs and snap frozen in a slurry of isopentane–dry ice. Pregnancy was confirmed in the last five mares by transrectal ultrasonography immediately before surgery. The samples were stored at –70°C. Tissue samples from a further six equine corpora lutea (two on days 40 and 41, two on days 20–30 of pregnancy and two from non-pregnant mares on day 10 of dioestrus) were snap frozen and stored at –70°C until protein extraction and immunoblotting.

Immunostaining procedures

Sections (6 µm) were immunostained using an avidin–biotin complex method described by Watson and Thomson (1996a). The primary antibody was raised in sheep against a peptide fragment (amino acids 82–107) of the predicted StAR protein sequence (Hartung *et al.*, 1995). The antibody was used at a dilution of 1:1500. The chromagen used (3-amino-9-ethylcarbazole) gave a red reaction product and the sections were counterstained with Meyer's haematoxylin. Negative control sections were included in which the first antibody was replaced with normal horse or sheep serum and controls of horse testis were included with each batch. Although the presence of StAR has not been demonstrated in equine testis, its presence in the Leydig cells of other species has been reported (Clark *et al.*, 1994).

Western blot analysis

Western blot analysis was performed on homogenates of equine corpora lutea to check the crossreactivity of the anti-bovine StAR antibody with equine StAR and to quantitate the amounts of protein present. Sheep adrenal gland was included as a positive control. Non-steroidogenic equine tissue (muscle) was included as a negative control. Western blotting was performed on 25 µg whole tissue homogenates of the equine corpus luteum, uterus, muscle and ovine adrenal gland. Briefly, each tissue was homogenized in five times (w/v) PBS containing 1% (w/v) sodium deoxycholate and 0.1% (w/v) SDS (homogenization buffer) on ice and centrifuged at 2000 *g* for 10 min to remove cell debris. After the protein concentration in the supernatant was determined according to Bradford (1976), all samples were diluted to 4 g l^{-1} with homogenization buffer. The samples were diluted 2:1 with sample loading buffer (35 mmol SDS l^{-1}, 1.4 mmol glycerol l^{-1}, 0.3 mmol 2-mercaptoethanol l^{-1}, 15 mmol bromphenol blue l^{-1}) and heat-treated at 90°C for 5 min before SDS-PAGE on a 12% (w/v) gel. After electrophoresis, the gel was washed in 25 mmol Tris buffer l^{-1} containing 192 mmol glycine l^{-1}, pH 8.3, at 20°C for 10 min and transferred to nitrocellulose paper. Transfer was carried out in the same buffer as described above, using a BioRad Miniblot cell system (BioRad Laboratories Ltd., Hemel Hempstead). The electrophoretic transfer of polypeptides to nitrocellulose paper was performed at 250 mA for 90 min at 10°C. The membrane was blocked overnight with Superblock blocking buffer (Pierce & Warriner, Chester), washed and incubated for 1 h with the polyclonal sheep antibody. The primary antibody dilution was 1:10.000 in PBS containing 3% (w/v) dried milk, 10% (v/v) Superblock and 0.05% (v/v) Tween 20. After sequential washing of the membrane with PBS plus 0.05% (v/v) Tween 20, visualization of the polypeptides that reacted with the primary antibody was achieved using a second (anti-sheep IgG) antibody labelled with horseradish peroxidase followed by enhanced chemiluminescence (Supersignal Ultra; Pierce & Warriner) according to the manufacturer's instructions. The bands illuminated were detected on X-ray film for visualization and by storage phosphor imaging system (Model GS-525, BioRad) for quantification.

Results

Small follicles were classified histologically as healthy ($n = 3$) or atretic ($n = 2$) on the basis of the criteria described by Kenney *et al.* (1979). Immunostaining for StAR protein was not present in a 1 mm growing follicle, but was present in the theca interna of

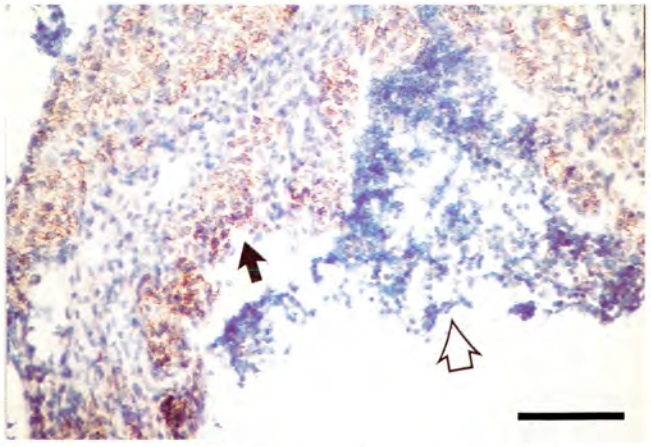

Fig. 1. Preovulatory follicle (40 mm) from an oestrous mare. Positive staining for steroidogenic acute regulatory (StAR) protein is present in the theca interna (solid arrow) but is not present in the granulosa layer (open arrow). Scale bar represents 100 μm.

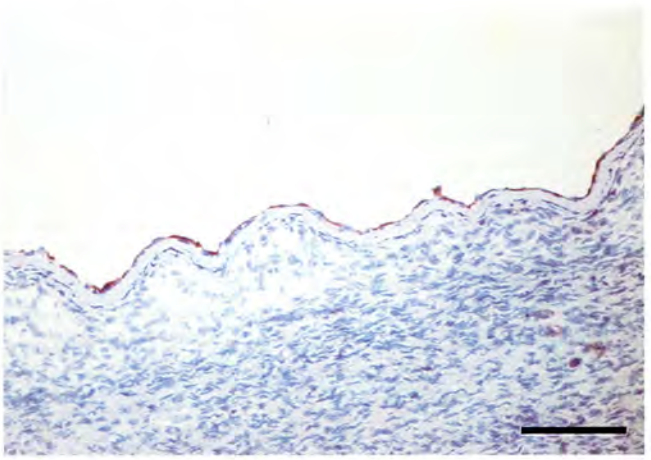

Fig. 2. Atretic equine follicle showing positive staining for steroidogenic acute regulatory (StAR) protein in the flattened cell layer lining the antrum. The separate identity of the thecal and granulosa cells has been lost and a thick basement membrane is present. Scale bar represents 100 μm.

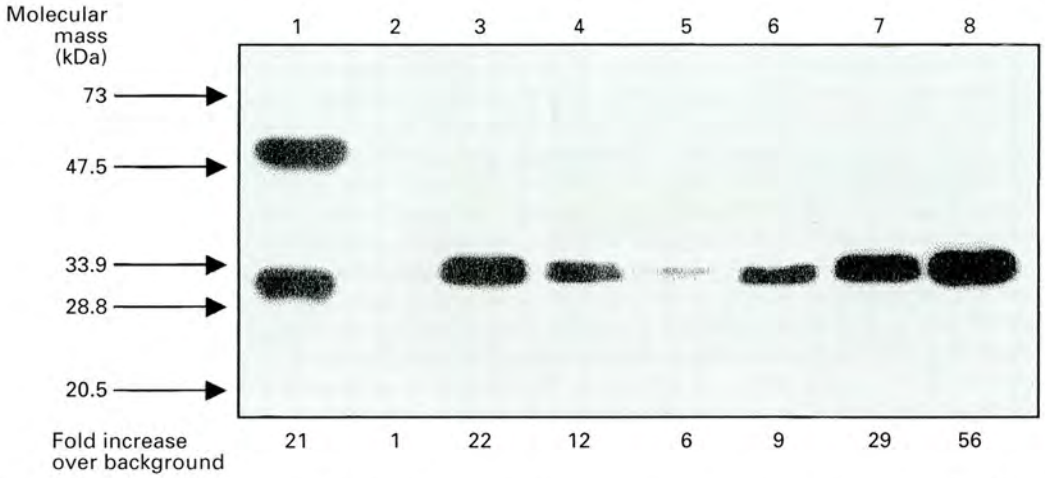

Fig. 3. Results of western blot analysis followed by phosphor imaging bands for steroidogenic acute regulatory (StAR) protein from sheep adrenal gland (lane 1), equine muscle (lane 2), mid-luteal corpora lutea from non-pregnant mares (lanes 3 and 4), corpora lutea from mares on days 20–30 of pregnancy (lanes 5 and 6) and corpora lutea from mares on day 40 or 41 of pregnancy (lanes 7 and 8). Positions of pre-stained molecular mass markers are indicated on the left-hand side.

follicles measuring 15–40 mm in diameter (Fig. 1). No staining was detected in the granulosa layer. Two preovulatory follicles (40 mm) recovered 24 h after hCG treatment also had staining of the cytoplasm, which was mainly confined to the theca, although some of the granulosa cells appeared to be positively stained. Immunostaining was present in the flattened granulosa cell layer of atretic follicles (Fig. 2).

Total protein extracted from six equine corpora lutea (two on day 40 or day 41 of pregnancy, two on days 20–30 of pregnancy and two from non-pregnant mares) had one band of approximately 30 kDa on the western blots (Fig. 3) and appeared to be of slightly higher molecular mass than StAR in the ovine adrenal gland. The amount of protein present was quantified by phospho-imaging of the western blots and was expressed as fold increase over background. The amounts in the two corpora lutea at day 40 or day 41 (29- and 56-fold) were higher than those in the two corpora lutea from non-pregnant mares (12- and 22-fold) and the two corpora lutea from days 20–30 of pregnancy (6- and 9-fold). StAR protein was not present in equine uterus or muscle, but was consistently present in ovine adrenal gland (positive control). The identity of the 50 kDa band present in the ovine adrenal gland is not known, but it is possible that because the StAR antibody was raised in sheep, it represents a non-specific crossreactant. Immunostaining of corpora lutea showed that the protein was confined to the large luteal cells (Fig. 4a–c). The intensity of the staining varied among cells. No staining was present in the trabeculae in which the small luteal cells are located. In the equine testis, staining was faint and was confined to the Leydig cells (Fig. 5). In sections in which the primary antibody was excluded, there was no evidence of endogenous peroxidase activity (Fig. 6).

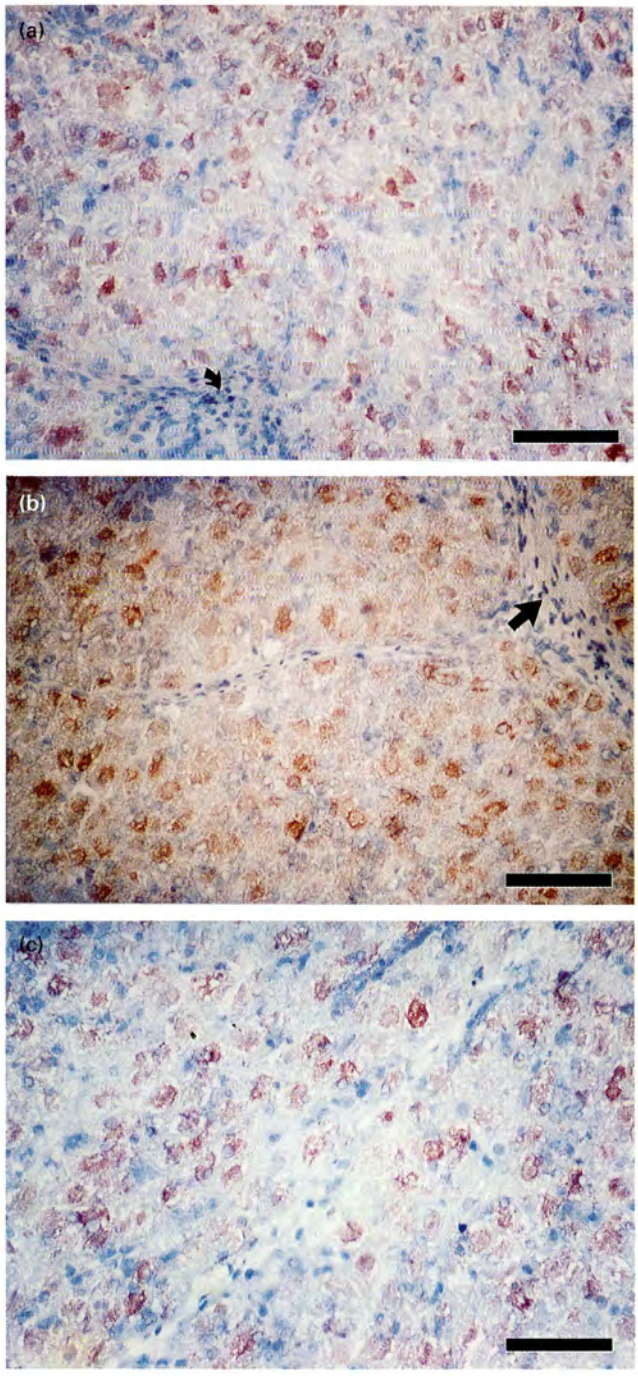

Fig. 4. Corpora lutea from a mare on (a) day 7 of dioestrus, (b) day 20 of pregnancy and (c) day 41 of pregnancy with positive staining for steroidogenic acute regulatory (StAR) protein in large luteal cells. Some cells stain much darker than others. No stained cells are observed in the trabeculae (arrows in a and b). Scale bars represent 100 µm.

Discussion

The results of the present study demonstrated the presence of a protein in the equine corpus luteum which migrated electrophoretically in a manner similar to that reported for StAR in mice (Clark *et al.*, 1994) and cows (Pescador *et al.*, 1996). The molecular mass appeared to be slightly higher

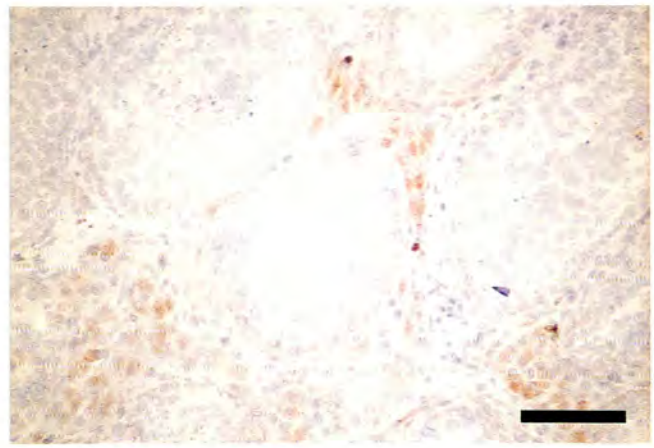

Fig. 5. Equine testis showing positively stained Leydig cells. Seminiferous tubules are negative. Scale bar represents 100 µm.

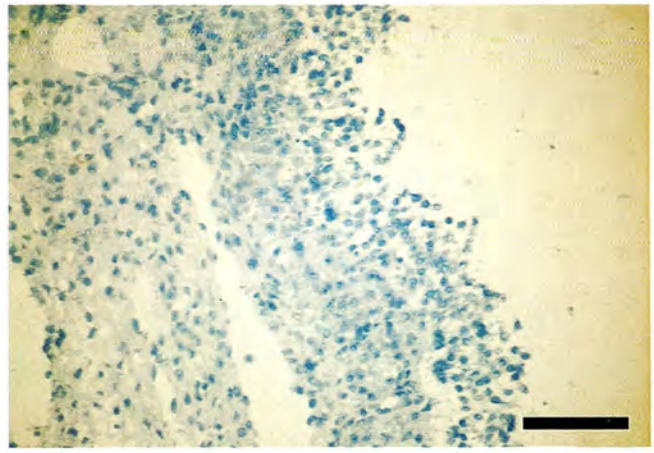

Fig. 6. Negative control section of equine follicle showed no positive staining. Scale bar represents 100 µm.

than that in sheep, but this minor inter-species variation is not unexpected. StAR is synthesized as a 37 kDa cytosolic precursor protein, which is then imported into mitochondria where it is transformed into four mature 30 kDa isoforms. The half-life of the 37 kDa precursor is very short (3–5 min) and the protein is not abundant in steroidogenic tissues (Epstein and Orme-Johnson, 1991; Stocco and Sodeman, 1991). It is thought that cholesterol is transported to the inner mitochondrial membrane during importation (Clark *et al.*, 1994; Juengel *et al.*, 1995). The inactive 30 kDa form, which remains associated with the mitochondrial membrane for some time, appears to be identified by immunoblotting.

The mRNA encoding the StAR protein has already been cloned and characterized from equine follicles (Kerban *et al.*, 1999) and is between 85 and 90% identical to StAR in other species. In the present study, StAR protein was confined to theca cells of healthy follicles, apart from the follicles obtained 24 h after hCG treatment. This is in agreement with expression of mRNA encoding StAR; StAR mRNA was detected in theca, but was undetectable in granulosa cells isolated before hCG treatment. Significant induction of StAR mRNA in granulosa cells was apparent only 30 h after hCG (Kerban *et al.*, 1999).

Therefore, it is likely that follicles in the present study were collected before mRNA encoding StAR was expressed in the granulosa layer. In bovine follicles, StAR mRNA appears to be restricted to the theca (Soumano and Price, 1997; Bao *et al.*, 1998), although in these studies the follicles were probably not collected just before ovulation and so a switch in mRNA StAR expression from theca to granulosa may still occur just before ovulation. Treatment with eCG increases accumulation of StAR mRNA in bovine follicles and it has been suggested that this reflects granulosa cell expression (Soumano and Price, 1997) as occurs in women undergoing ovarian hyperstimulation (Sugawara *et al.*, 1995). Similarly, in mares, it is likely that the LH-like activity of hCG stimulated expression of StAR in granulosa cells of the equine follicle.

In the present study, the flattened antral cell layer of late atretic follicles showed immunostaining for StAR. Bao *et al.* (1998) reported StAR mRNA in late atretic bovine follicles and suggested that luteinization of granulosa cells after degeneration of the oocyte might result in StAR mRNA expression.

In the equine corpus luteum, immunostaining for StAR was confined to the large luteal cells, which are presumed to be luteinized granulosa cells (Harrison, 1946; Van Niekerk *et al.*, 1975), and was not detectable in the trabeculae which contain the small cells. This corresponds well with the late marked decrease in StAR mRNA in granulosa cells just before ovulation (Kerban *et al.*, 1999). No differences in distribution of immunostaining for StAR were noted in corpora lutea from non-pregnant mares, or from corpora lutea of pregnant mares before (day 20) or after (day 40+) onset of eCG secretion from the endometrial cups, although there were differences in staining intensity among the large cells. Similar differential staining has been described for aromatase in equine corpora lutea (Watson and Thomson, 1996b). Concentrations of eCG in blood were not measured in the present study, but the onset of eCG secretion between day 36 and day 40 of pregnancy is well established (Allen and Stewart, 1992), and another study demonstrated increased expression of $P450_{C17}$ protein in the same corpora lutea collected after day 40 of pregnancy (Rodger *et al.*, 1998), which appears after the onset of eCG secretion (Albrecht *et al.*, 1997). It was hypothesized that eCG might upregulate StAR expression in the equine corpus luteum, as occurs in bovine follicles (Soumano and Price, 1997). In the limited number of samples examined in the present study, StAR did appear to be more abundant in corpora lutea after day 40 of pregnancy. The increase in StAR may have regulated luteal oestrogen production by stimulating cholesterol transport to provide substrate for androgen and therefore oestrogen synthesis. Further work needs to be performed on a larger number of mares at different stages of pregnancy and after eCG supplementation to confirm the results of this study.

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Journal of Reproduction and Fertility (1996) **108**, 239–244

Immunolocalization of aromatase P-450 in ovarian tissue from pregnant and nonpregnant mares and in ovarian tumours

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Aromatase P-450 ($P\text{-}450_{arom}$) is a crucial regulatory enzyme that is necessary for conversion of androgens to oestrogens. Corpora lutea and follicles were obtained from the ovaries of cyclic mares and from mares at day 20 and days 40–70 of pregnancy. The presence of $P\text{-}450_{arom}$ within specific cell types was investigated by immunostaining to determine potential sites of oestrogen synthesis. Immunoreactivity for $P\text{-}450_{arom}$ was confined to the granulosa layer of non-atretic follicles > 5 mm in diameter and to corpora lutea at all stages of the oestrous cycle and during pregnancy. These findings confirm that aromatization of androgens occurs within the granulosa cells of the preovulatory follicle of the mare and that the corpus luteum of the mare has the capacity for oestrogen production if adequate androgen substrate is available. Granulosa cells in ovarian tissue from three mares with granulosa cell tumours showed little staining for $P\text{-}450_{arom}$, which suggests that these tumours have little aromatizing capacity.

Introduction

The mechanism of steroidogenesis by follicles and corpora lutea in mares remains contentious. Originally it was reported that the primary steroidogenic role of granulosa cells in the preovulatory follicle was to produce progesterone, while the theca interna was responsible for oestradiol production (Short, 1962; Channing and Grieves, 1969; Younglai, 1971). The corpus luteum was not thought to produce oestrogen (Short, 1964; Mahajan and Samuels, 1974). However, it was also found that, although granulosa and luteal cells had only a limited capacity to produce oestradiol *de novo*, they could aromatize androgens to oestradiol *in vitro* (Ryan and Short, 1965; Mahajan and Samuels, 1974; Al-Timimi *et al.*, 1989). Interaction between cultured granulosa and theca cells from mare follicles was found to be necessary for oestrogen synthesis, and it was then suggested that androgens of thecal origin were aromatized to oestradiol by granulosa cells (Sirois *et al.*, 1991). Although the corpus luteum of the nonpregnant mare is not thought to produce oestrogen (Short, 1964; Mahajan and Samuels, 1974), recent studies *in vivo* have provided evidence that corpora lutea from mares that are between 35 and 70 days of pregnancy act as a source of oestrogen (Daels *et al.*, 1991).

Granulosa cell tumour is the most common neoplasm of the mare ovary and represents 2.5% of all equine neoplasms (Sundberg, 1977). These neoplasms are frequently accompanied by behavioural abnormalities that are manifest as anoestrus, nymphomania or stallion-like behaviour. Testosterone concentrations are often increased in mares exhibiting aggressive male-type behaviour and comprise approximately 30% of affected mares (Stabenfeldt *et al.*, 1979). However, concentrations of oestradiol in mares with granulosa cell tumours are variable and are often not related to the behavioural changes (Stabenfeldt *et al.*, 1979). It has been proposed that aromatization of testosterone to oestradiol may be low in affected mares, as mares with very high concentrations of circulating testosterone do not necessarily have concomitantly high oestradiol concentrations (Stabenfeldt *et al.*, 1979). However no studies have determined the aromatase activity of equine granulosa cell tumours.

The development of antibodies against various steroidogenic enzymes has allowed detailed examination of ovarian steroidogenesis in other species, but to date there have been no studies in the mare ovary on the expression of steroidogenic enzymes at the cellular level throughout the oestrous cycle and pregnancy. In the ovarian steroidogenic pathway, aromatase cytochrome P-450 ($P\text{-}450_{arom}$) is a crucial regulatory enzyme that is necessary for conversion of androgens to oestrogens. In the present study, ovarian tissue was obtained from mares at various stages of the oestrous cycle, during early pregnancy, and from three mares with granulosa cell tumours, and stained immunohistochemically to detect and localize the cells capable of aromatization.

Materials and Methods

Follicles and corpora lutea were obtained from 20 pony mares aged 4–18 years and weighing 250–380 kg. The stage of cycle was monitored by daily ultrasonographic examination of the ovaries and uterus. Day of ovulation was designated day 0. Ovaries were removed by a colpotomy incision as described by Watson and Sertich (1990). Neuroleptanalgesia was induced

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by intravenous administration of romifidine (50 μg kg^{-1}; Sedivet, Boehringer Ingelheim Ltd, Bracknell, Berks) and butorphanol (25 μg kg^{-1}; Torbugesic, Willows Francis Veterinary Ltd, Crawley, Surrey). Flunixin meglumine (1 mg kg^{-1}; Finadyne, Schering-Plough Animal Health Ltd, Watford, Herts) was administered immediately after surgery. Corpora lutea were obtained from between two and six mares in early (days 2–3), and mid- (days 7–12) dioestrus, in the early follicular phase (day 16), 1 day after administration of the prostaglandin $F_{2\alpha}$ analogue cloprostenol (263 μg; Estrumate, Mallinckrodt Veterinary Ltd, Uxbridge, Middlesex) in mid-dioestrus, and during pregnancy (days 20 and days 40–70). Preovulatory follicles were obtained during oestrus ($n = 7$) and smaller follicles were dissected out of the ovary during dioestrus ($n = 5$). A jugular blood sample was collected from all mares before surgery and the plasma was stored at −20°C until assayed for progesterone. Granulosa cell tumours were obtained from a further three mares by midline laparotomy under general anaesthesia. Serum concentrations of testosterone and oestradiol were determined by a commercial laboratory (SCL Bioscience Services Ltd, Cambridge) in samples taken before surgery from these three mares.

Tissue was embedded in OCT compound (Miles Inc., Elkhart, IN) on cork disks and snap frozen in a slurry of isopentane–dry ice. The samples were then stored at −70°C.

Immunostaining procedures

Sections (6 μm) were immunostained using an avidin–biotin complex method described by Watson and Thomson (1996). The primary antibody, which was kindly provided by E. Simpson (University of Texas), was raised in rabbits against synthetic aromatase cytochrome P-450, and used at a final concentration of 50 μg ml^{-1}. The chromagen used (AEC; 3-amino-9-ethylcarbazole) produced a red reaction product and the sections were counterstained with Meyer's haematoxylin.

Negative control sections in which the first antibody was replaced with normal horse serum, and positive controls of horse testis, were included with each batch of samples.

Assay of plasma progesterone

Progesterone concentrations were determined in unextracted plasma using a radioimmunoassay kit (ICN Biomedicals Inc., Costa Mesa, CA) employing an ^{125}I-progesterone tracer (Watson *et al.*, 1995). The limit of detection of the standard curve was 0.25 ng ml^{-1} and the inter- and intra-assay coefficients of variation were 6.0 and 6.8%, respectively.

Results

Circulating progesterone concentrations in all mares were > 1 ng ml^{-1} except for mares in early follicular phase and on the day after treatment with prostaglandin $F_{2\alpha}$.

Immunoreactivity for P-450$_{arom}$ was confined to the granulosa cells of non-atretic follicles > 5 mm in diameter (Fig. 1). The granulosa cells of small follicles < 5 mm in diameter and the lining of atretic follicles did not stain. No staining for P-450$_{arom}$ was detected in the thecal cells, stromal cells or preantral follicles.

Fig. 1. Immunoreactivity of P-450$_{arom}$ in a 30 mm preovulatory follicle taken from a mare's ovary. Note localization of staining in granulosa cells (arrow). Sections were counterstained with Meyer's haematoxylin. Scale bar represents 10 μm.

The early corpus luteum showed many strongly staining luteinizing granulosa cells (Fig. 2a). After structural reorganization of the corpus luteum in mid-dioestrus, there was overall weak immunoreactivity with patchy, strongly stained large cells (Fig. 2b). Cells within the trabeculae did not show staining. On day 16, when the corpora lutea started to show structural changes associated with luteolysis, they still retained the same staining pattern as in mid-dioestrus. This pattern also persisted in corpora lutea obtained 24 h after administration of cloprostenol. These staining patterns were consistently present in replicate sections stained on different days.

On day 20 of pregnancy, uniformly pale immunostaining was present. Dark-staining cells were not apparent (Fig. 3a), but by days 40–70 dark staining cells were again obvious (Fig. 3b). In one secondary corpus luteum removed on day 50 that had ovulated 10 days previously, patches of darkly staining non-luteinized granulosa cells were seen.

Two of the mares with granulosa cell tumours had low testosterone and oestrogen concentrations (testosterone = 0.28 and 0.06 nmol l^{-1}, oestradiol = < 20 pmol l^{-1}) and one had increased concentrations (testosterone = 1.0 nmol l^{-1}, oestradiol = 230 pmol l^{-1}). Granulosa cells from the tumours of the mares with low testosterone showed very little staining for P-450$_{arom}$. Only occasional individual cells were strongly positive (Fig. 4). In the mare with increased concentrations of blood testosterone and oestradiol, occasional groups of granulosa cells showed positive staining.

In sections in which the primary antibody was excluded, there was no evidence of endogenous peroxidase activity (Fig. 5a). In positive controls (testis), interstitial cells stained, whereas no staining was observed in the seminiferous tubules (Fig. 5b).

Discussion

This is the first report of immunolocalization of P-450$_{arom}$ in equine ovarian tissue. A homologous antibody for equine P-450$_{arom}$ was not available, but aromatase is known to have

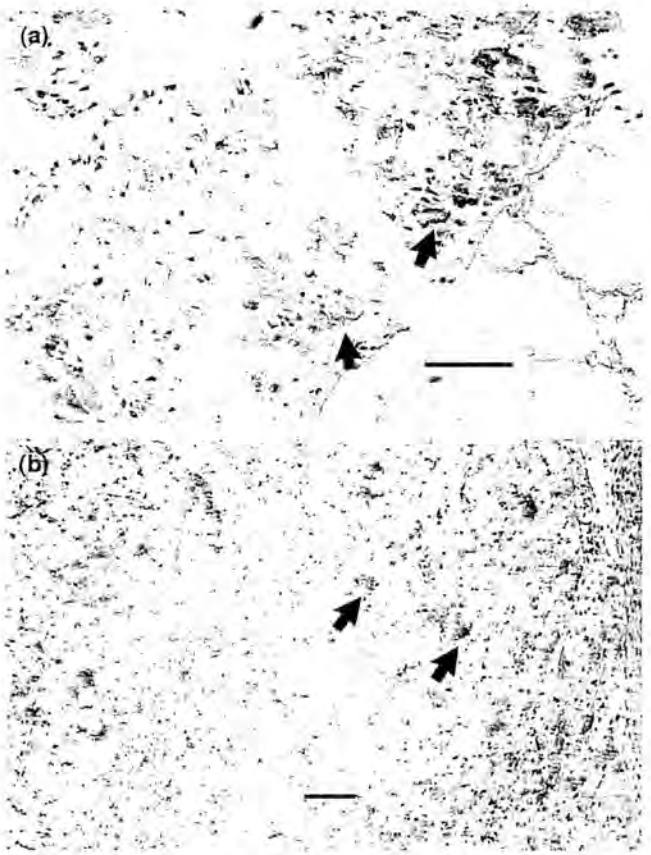

Fig. 2. Immunoreactivity of P-450$_{arom}$ in (a) the corpus luteum taken from the ovary of a mare at day 2 of the oestrous cycle. Luteinizing granulosa cells are strongly stained (arrows); and (b) corpus luteum at day 10 of the oestrous cycle. Note the patchy, strong staining localized to individual cells (arrows). Sections were counterstained with Meyer's haematoxylin. Scale bars represent 10 μm.

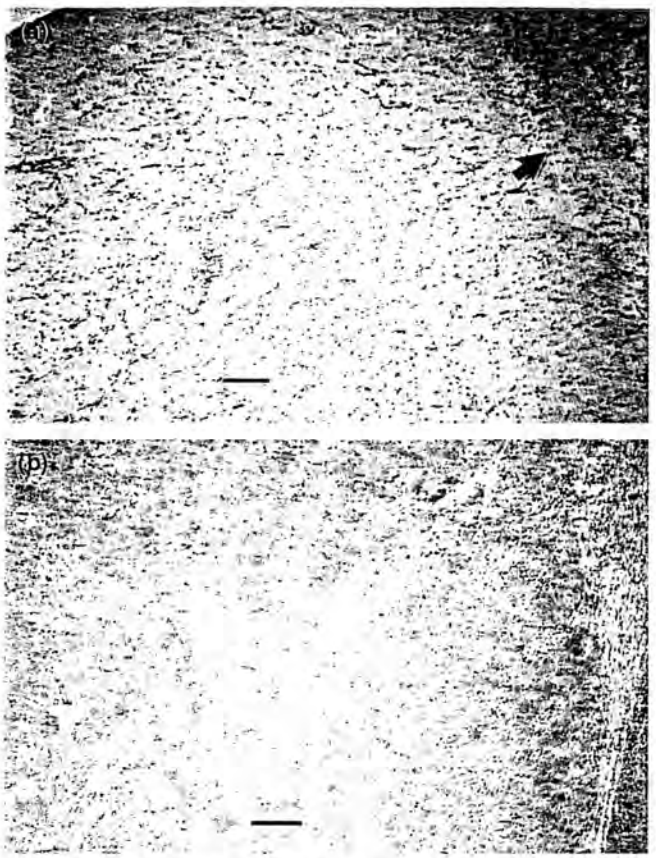

Fig. 3. Immunoreactivity of P-450$_{arom}$ in (a) the corpus luteum taken from the ovary of a mare at day 20 of pregnancy. The large cells show uniform weak immunostaining. Note the unstained trabecula (arrow). (b) The corpus luteum at day 70 of pregnancy. Individual large cells are strongly stained. Sections are counterstained with Meyer's haematoxylin. Scale bars represent 10 μm.

wide sequence homology among different species (Simpson *et al.*, 1994) and a heterologous antibody has previously been found to recognize interstitial cells in stallion testes (Eisenhauer *et al.*, 1995). In fact, stallion testis was used as the positive control in our study and staining was confined to interstitial cells as previously reported (Eisenhauer *et al.*, 1995).

Immunoreactivity to P-450$_{arom}$ was present in the granulosa cells of medium to large ovarian follicles but was absent in small follicles (< 5 mm). Measurement of steroid concentrations in follicular fluid from mares has shown that highest concentrations are present in large vascularized follicles, with only low concentrations present in small follicles (Short, 1961; Van Rensburg and Van Niekerk, 1968; Channing and Grieves, 1969). This finding is in agreement with the lack of enzyme immunoreactivity in small follicles in our study. Atretic follicles, in which the separate identity of the theca and granulosa cells was lost, showed no evidence of immunoreactive P-450$_{arom}$. Concentrations of oestrogen are very low in these follicles (Kenney *et al.*, 1979). Results from cell culture experiments and analysis of steroid content of follicular fluid have led to the conclusion that the theca interna is responsible for oestrogen synthesis in the preovulatory follicle of the mare (Short, 1962; Channing and Grieves, 1969; Younglai, 1971; Hay *et al.*, 1975). However more recent studies have suggested

Fig. 4. Immunoreactivity of aromatase P-450 in granulosa cells taken from the ovary of a mare with a granulosa cell tumour. Only a few cells showed strong positive staining (arrows). Scale bar represents 10 μm.

that testosterone is the main product of cultured equine thecal cells, and that androgens of thecal origin may be aromatized to oestradiol by granulosa cells (Sirois *et al.*, 1991; Tucker *et al.*,

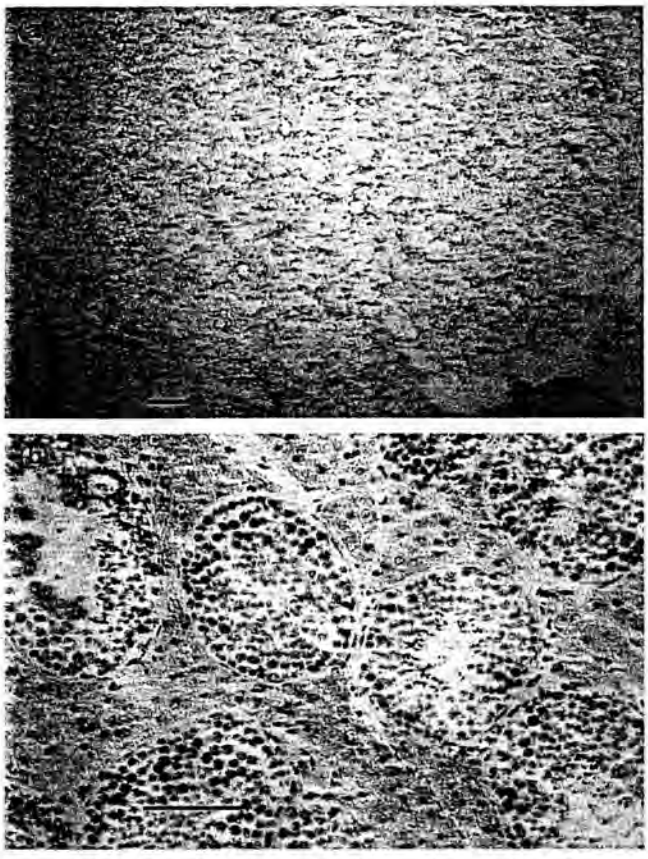

Fig. 5. (a) Negative control section of corpus luteum from the ovary of a mare in which the primary antibody for aromatase P-450 was excluded. (b) Positive control section of stallion testis. No immunostaining for aromatase P-450 was present in the seminiferous tubules, whereas the surrounding interstitial cells stained positive. Scale bars represent 10 μm.

1991). Culture of isolated cell types can be subject to contamination with other cell populations (Ryan and Short, 1965) and to changes that can transform cells *in vitro* such as the propensity for granulosa cells to luteinize *in vitro* (Channing, 1969). *In situ* detection of enzyme by immunohistochemical staining does not allow quantitation of enzyme content, but does localize the enzymes to specific cell types. The results of the present study would indicate, therefore, that aromatization of androgens is confined to granulosa cells within the follicle.

Most studies agree that oestradiol is not present in the corpus luteum of the nonpregnant mare (Short, 1964; Mahajan and Samuels, 1974); however, luteal cells and microsomes from corpora lutea appeared to be capable of aromatizing testosterone and androstenedione when these were provided as substrates (Mahajan and Samuels, 1974; Al-Timimi *et al.*, 1989). Furthermore, the presence of oestradiol in the mid-luteal corpus luteum was reported by Younglai (1971) and there is unpublished circumstantial evidence that oestrogen secretion increases during the mid-luteal phase and decreases rapidly after administration of $PGF_{2\alpha}$ (cited in Daels *et al.*, 1991). We have shown that immunoreactive P-450_{arom} is present in luteal cells throughout dioestrus. Therefore, the potential for oestrogen production resides within the corpus luteum of the cyclic mare. Although thecal cells are present in the trabeculae of the mare corpus luteum (Harrison, 1946), and small luteal cells that might be expected to be of thecal origin are steroidogenic (Kelly *et al.*, 1988; Broadley *et al.*, 1994), the small thecal-derived cells are vastly outnumbered by the large granulosa-derived cells. As the granulosa-derived cells appear to depend on the theca-derived cells for androgen substrate, the ability of the corpus luteum to produce androgens and oestrogens may be directly related to the extent to which thecal cells contribute to the corpus luteum (Henderson and Swanston, 1978). However, there appear to be marked differences in steroidogenic capacities of ovarian cells among species (Hammerstein *et al.*, 1964; Watson and Leask, 1975; Al-Timimi *et al.*, 1989; Lautincik *et al.*, 1994). Most studies have shown that the corpus luteum of the non-pregnant mare does not produce oestrogen. If this is the case, high concentrations of circulating progesterone during dioestrus could inhibit aromatase activity in the mare ovary (Amri *et al.*, 1993) as has been proposed in sows (Gregoraszczuk, 1994) and rats (Fortune and Vincent, 1983). Therefore, factors other than cell type may influence luteal oestrogen production.

In the mare corpus luteum, immunostaining for P-450_{arom} was present shortly after luteolysis, whereas the human corpus luteum demonstrates neither P-450_{arom} immunoreactivity nor mRNA expression after luteal regression (Suzuki *et al.*, 1993). However the corpora lutea examined by Suzuki *et al.* (1993) were probably older than the mare corpora lutea in the study reported here. The presence of immunoreactivity for aromatase in the mare corpora lutea in the period immediately after luteolysis, when steroid production ceases, suggests that cessation of steroidogenesis is dependent on mechanisms other than disappearance of aromatase. It is thought that $PGF_{2\alpha}$ may cause luteolysis in domestic species by inhibition of second messenger pathways (Fletcher and Niswender, 1982) rather than by having a direct effect on steroidogenic enzymes.

The mare corpus luteum appears to comprise at least two distinct populations of large luteal cells. Some individual cells stained very heavily for aromatase, whereas the staining in surrounding cells was much weaker. Apparent differences in staining intensities within cell populations have similarly been reported in granulosa cells of preovulatory follicles of rats and mice (Ishimura *et al.*, 1989), interstitial cells in stallion testes (Eisenhauer *et al.*, 1995), and in theca interna cells in follicles of sheep and cattle (Conley *et al.*, 1995), leading to suggestions that there may be functional differences within these populations. Although immunostaining is not a quantitative technique, a significant correlation has been shown between immunointensity for aromatase P-450, measured by computerized image analysis, and enzyme activity (Sasano, 1994). During early pregnancy the difference in staining between cells in the mare corpus luteum was less marked, whereas by day 40, strong areas of staining had returned.

It seems likely that equine chorionic gonadotrophin (eCG), the concentration of which increases around day 35 of pregnancy, acts as the trigger for oestrogen production by the primary corpus luteum. Recent work has shown that treatment with eCG stimulates oestrogen secretion in pregnant mares with a corpus luteum, but not in those in which the corpus luteum has been removed (Daels and Albrecht, 1995). Luteal mRNA encoding aromatase P-450 in the mare corpus luteum

was lower after the onset of eCG secretion than at any other stage during pregnancy, despite the increased oestrogen secretion (Albrecht *et al.*, 1995). This observation led to the suggestion that the increase in luteal oestrogen secretion is due to post-transcriptional regulation of aromatase activity by eCG (Albrecht *et al.*, 1995). However, there is usually a good correlation between concentrations of mRNA encoding aromatase P-450 and amount of enzyme protein (Doody *et al.*, 1990). The results reported here show the appearance of darker staining in certain individual cells after the onset of eCG secretion, which might indicate that these cells were producing more aromatase than earlier in pregnancy, but the pattern and intensity of staining was not different from that shown by corpora lutea from non-pregnant mares. These data suggest that P-450$_{17\alpha}$ activity, which provides the androgen substrate, may be the important limiting factor in secretion of oestrogen by the corpus luteum of pregnant mares. The appearance of P-450$_{17\alpha}$ in luteal cells may be stimulated by eCG. Indeed P-450$_{17\alpha}$ has been induced in thecal cells of immature rats by treatment with eCG and hCG (Ishimura *et al.*, 1990), and injection of rats with eCG increased total hybridizable transcript for P-450$_{17\alpha}$ (Doody *et al.*, 1991). We have preliminary evidence that P-450$_{17\alpha}$ is present in the corpus luteum during pregnancy but not during the oestrous cycle (Rodger *et al.*, 1995). Similarly a previous report has described low 17,20 lyase activity of corpora lutea from non-pregnant mares as demonstrated by the poor capacity of luteal tissue to metabolize progesterone to androgens (Mahajan and Samuels, 1974), although another study showed that low concentrations of androgens were produced (Al-Timimi *et al.*, 1989).

The biological significance of any oestrogen secretion by the primary corpus luteum is questionable as it is well documented that mares ovariectomized on day 34 or 35 of pregnancy and supplemented with only progestagens can maintain a pregnancy until placental steroid production takes over at around day 100 (Shideler *et al.*, 1982).

Ovarian tumours are relatively common in mares and account for approximately 5.6% of all neoplasms (Righ *et al.*, 1985). By far the commonest type of ovarian neoplasm is the granulosa cell tumour (McEntee, 1990). The failure of the tissue from two of the granulosa cell tumours to show significant immunostaining and the low level of staining in the other is interesting and is similar to immunostaining of human granulosa cell tumours (Sasano, 1994). The high circulating oestrogen concentrations present in some women with granulosa cell tumours (Besch *et al.*, 1966) are not characteristic of this tumour in mares (Stabenfeldt *et al.*, 1979). However, the tumour from the mare that had higher concentrations of circulating oestradiol showed a greater degree of immunostaining. It has been suggested that aromatization of testosterone to oestradiol may be low in granulosa cell tumours (Stabenfeldt *et al.*, 1979) and our results showed that very little aromatase was present.

The results from the present study showed good agreement between expression of P-450$_{arom}$ and recent reports of steroidogenesis by isolated cell types *in vitro*. Together with our previous results showing staining for P-450$_{17\alpha}$ in theca interna cells of preovulatory follicles (Rodger *et al.*, 1995), we have confirmed that the two-cell theory of steroidogenesis, in which thecal androgen is aromatized by granulosa cells (McNatty *et al.*, 1979; Hillier, 1981), applies to the mare ovary.

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ELSEVIER

IMMUNOLOCALIZATION OF P450C17 IN THE MARE CORPUS LUTEUM

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ABSTRACT.

Although the mare corpus luteum (CL) is capable of aromatization, the expression of other enzymes involved in estradiol synthesis is not yet clear. This study examined the localization of P450C17 in the mare CL at different stages of its functional development. In ovaries from follicular phase mares P450C17 was localized in the theca cells of ovarian follicles. Following ovulation, no immunostaining for P450C17 was detected in the mature CLs of nonpregnant mares. In pregnant mares, no immunostaining for P450C17 was identified in the corpus luteum prior to secretion of eCG by the feto placental unit at Day 35 of pregnancy. The P450C17 was found to be expressed in CLs retrieved from Day 40 of pregnancy onwards. The changing expression of P450C17 raises the possibility that this may be a regulatory step for estrogen synthesis in the mare ovary.

Key words: ovary, mare, steroidogenesis, estrogen

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mm in size were dissected. CL were obtained in a similar manner from 2,2,2,2, and 2 mares on Days 2, 7,12 (day of ovulation = Day 0) of the luteal phase and on Days 20 and 40 of pregnancy, respectively, as well as from 4 mares on Day 70 of pregnancy. Immediately prior to ovariectomy blood was collected from the jugular vein into heparinized containers for progesterone estimation. Progesterone concentrations were measured in unextracted plasma using a commercial kit (ICN Biomedical Inc; Costa Mesa, CA);(15). The presence of a functional corpus luteum was confirmed in all cases by a circulating progesterone concentration of more than 1ng/mL.

Ovaries were sliced, fixed in 4% paraformaldehyde for 24 h and then mounted in a paraffin block. To investigate further the relationship between P450C17 immunolocalization and steroidogenesis in the mare, additional ovaries were obtained from 6 mares undergoing ovariectomy for granulosa cell tumours; the ovarieswere fixed in paraformaldehyde as above, and immediately prior to ovariectomy venous blood was collected into heparinized containers and subjected to radioimmunoassay for estrogen and testosterone by a commercial laboratory (SCL Bioscience Services Ltd; Cambridge, UK)

Testes were obtained at postmortem from 3 stallions for use as positive control tissue for P450C17 immunostaining. Equine testes produce testosterone, and P450C17 is involved in steroidogenesis in this tissue. Small pieces of testis were fixed in paraformaldehyde in the same manner as ovarian tissue.

Immunohistochemistry

Sections (5μm) were cut from paraffin blocks, mounted on glass slides, air dried at 56^{0}C, dewaxed in xylene then rehydrated in 100%, 95% and 70% ethanol. The sections were then preabsorbed for 20 min with normal goat serum diluted 1:5 in tris buffered saline.

A polyclonal antibody, raised in rabbits against human P450C17 (gift of Professor M. Waterman, Vanderbilt University, Nashville, TN), was applied directly onto sections at 1:500 dilution. Optimal antibody dilutions and incubation times had been deduced from initial experiments using equine testes as a positive control tissue. Sections were incubated in a humidified chamber at 4°C for 20 h. Biotinylated goat anti-rabbit secondary antibody (Dako; High Wycombe, UK) at a dilution of 1:500 was then applied for 30 min. Colouring was obtained with either nitroblue tetrazium chloride (NBT) by means of an avidin-biotin reaction with alkaline phosphatase, or with Vector red (Vector; Peterborough, UK). Sections were then counterstained with haematoxylin. No difference was observed in the level of staining using the 2 different chromogens. Negative staining control sections were incubated with normal rabbit serum (Dako) at 1:750 dilution in place of P450C17 primary antibody. Granulosa cell tumour sections (5 μm) and sections of equine testis (5 μm) were processed as above.

RESULTS

Steroid Radioimmunoassay

Serum progesterone levels of >1ng/mL were detected in all mares except those in estrus.

High levels of serum testosterone (>1nmol/L) and estrogen (>200pmol/L) were detected in 2 mares with granulosa cell tumours. The remaining 4 mares had serum testosterone levels below 0.3nmol/L and serum estrogen levels below 20pmol/L. Limit of detection for estradiol was 20pmol/L and for testosterone was 0.05nmol/L. Estradiol assay cross reactivity was 10% for estrone and <1% for other

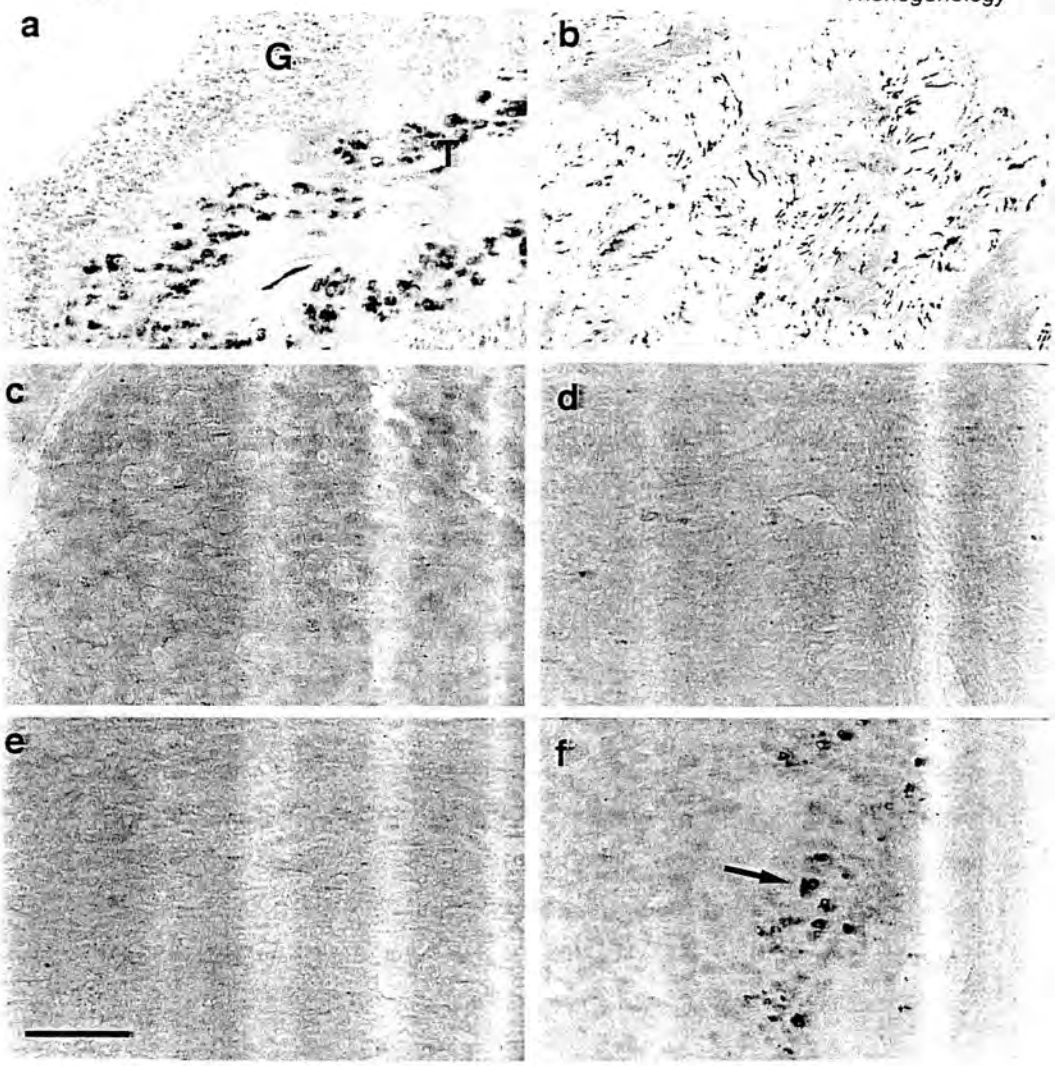

Figure 1. Immunostaining with nitroblue tetrazium chloride for P450C17 in the mare ovary (x400). the scale bar indicates 100μm. in the preovulatory follicle the enzyme was localized in theca (T) but not granulosa (G) cells (a). In the CL of the nonpregnant mare (b) P450C17 was seen on Day 2 (ovulation=Day 0), but not on Day 7 (c) or Day 12 (d). Sections incubated with rabbit serum in place of primary anti- P450C17 show no stain (negative control; e).

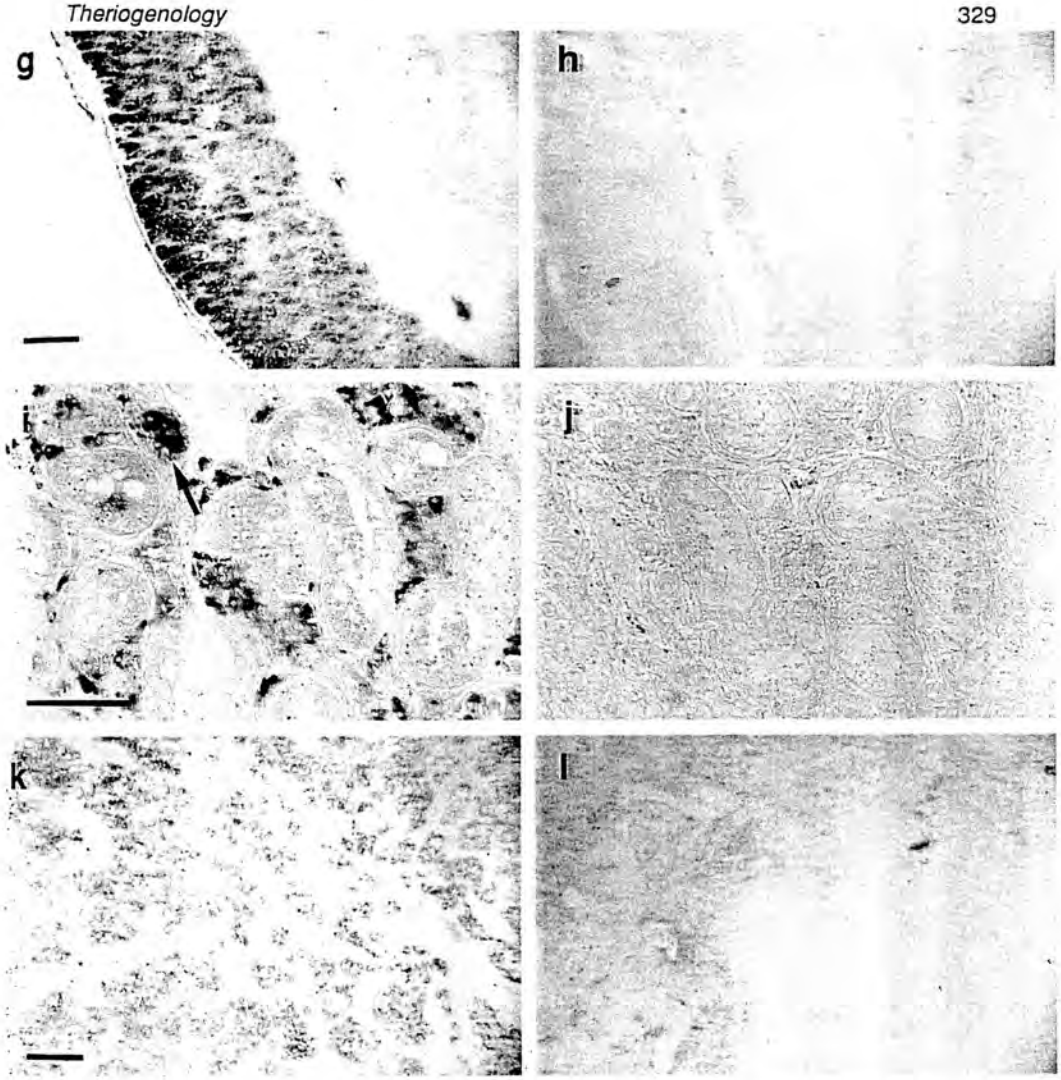

Figure 1. In the pregant mare on Day 70, P450 immunostaining was seen on the periphery of the CL (f; arrow) or as a continuous band (g). Equine testis (positive control) shows the enzyme within the Leydig cells (i; arrow). Tissue from a granulosa cell tumour which was associated with high or low plasma testosterone stained positive (k) or negative (l) for P450C17, respectively. Sections of pregnant Day 70 CL (h) and equine testes (j) incubated with rabbit serum in place of anti-P450C17 show no stain.

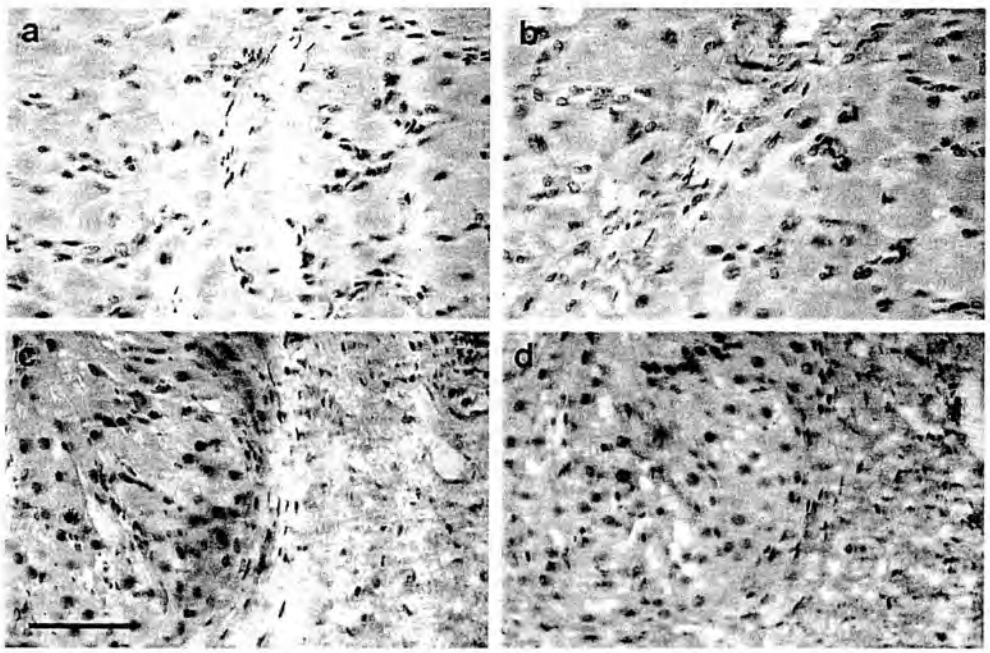

Figure 2. Immunostaining with Vector red for P450C17 before and after eCG secretion. The scale bar indicates 100μm. P450C17 is not expressed in the gland at Day 20 of pregnancy (a), but is present in the gland at Day 40 (c). Micrographs (b) and (d) represent negative control sections incubated with rabbit serum in place of primary antibody for (a) and (c) respectively.

localization appears between Days 20 and 40 of pregnancy, suggesting that this appearance may be triggered by secretion of eCG from the feto-placental unit, as this hormone first appears in the circulation at this time. Daels et al. (7) have recently demonstrated a close temporal relationship between the onset of eCG secretion and luteal testosterone secretion in mares. the level of aromatase expression is well conserved between CLs of different ages and through changing hormonal environments (14). Consistent with our findings, recent studies (1) have shown an upregulation of P450C17 mRNA expression in the mare CL after eCG secretion. The possibility thus arises that the presence of P450C17 is the critical regulatory step in early pregnancy, at which estrogen secretion is controlled in the mare CL.

Expression of P450 is not an absolute requirement for estradiol synthesis since androgen substrates for aromatase may be obatined from other organs via the circulation. However, since ovariectomized mares are not capable of testosterone secretion even when stimulated with ACTH (12), the circulationg androgens are not likely to play a major role in estrogen synthesis in the mare.

Variation was observed in the distribution of immunohistochemical staining of cytochrome P450C17 in CLs from pregnant mares. Although most CLs exhibited peripheral P450C17 staining, one gland showed a different pattern with a band of intense staining surrounded by negative tissue. It may be that this was an accessory CL, or luteinized unruptured follicle. The pattern of enzyme expression in accessory CLs may bear more resemblance to that of the follicle than that of the CL.

The P450C17 was detected in only the granulosa cell tumours which secreted high levels of testosterone and estradiol. This may be a further indication that localization of P450C17 is related to the steroidogenic capacity of a tissue.

This is the first report of the immunohistochemical localization of the enzyme P450C17 in the CL of the mare. Our data demonstrates that P450C17, an enzyme necessary for the synthesis of 17β-estradiol, is present in the follicles and in the CL of pregnancy of the mare. In contrast, this enzyme is not present in the mature nonpregnant mare CL or in the pregnant mare prior to eCG secretion. The changing pattern of P450C17 observed in the present study suggests that this enzyme may have a role in regulating estradiol synthesis in the mare ovary.

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ELSEVIER

COMPARTMENTALIZATION OF STEROIDOGENESIS BY THE EQUINE CORPUS LUTEUM

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ABSTRACT

The presence of cytochrome $P450_{C17}$ within equine follicles and corpora lutea (CL) was detected by immunostaining. Two different antibodies were used which had previously been shown by immunoblotting to cross-react with equine $P450_{C17}$. Strong positive immunostaining was present in the theca-derived cells of the CL during the estrous cycle and pregnancy. In the CL from mares after Day 40 of pregnancy there were also occasional bands of positively stained cells which resembled the polyhedral-shaped theca cells seen in preovulatory follicles. The pattern of immunostaining suggested compartmentalization of steroidogenesis within the equine CL with small cells possessing the potential to produce androgen which could then be aromatized to estrogen by the large luteal cells

Key words: mare, corpus luteum, steroidogenesis

INTRODUCTION

Unlike most other domestic species, the CL of the mare is capable of producing estrogen. Although the CL of the nonpregnant mare will synthesize estrogen in vitro when appropriate androgen substrate is provided (4,13), it appears not to synthesize estrogen in vivo (13,20). During pregnancy, however, after the onset of eCG secretion from the endometrial cups at around Days 35 to 40, increases in circulating estrogen and androgen result from luteal steroidogenesis (7,8). It seems that in the equine CL, estrogen is produced via the Δ^4 steroidogenic pathway (1,2). Therefore, cytochrome $P450_{C17}$ is responsible for metabolizing progesterone to androgen, the substrate for aromatization. Cytochrome $P450_{arom}$ is present in the equine CL both in the cyclic mare and during pregnancy (1,24), and, therefore, the potential exists for estrogen synthesis even in the

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nonpregnant mare. The change in steroidogenesis which occurs at the onset of eCG secretion in the pregnant mare has been directly attributed to the effect of eCG (7,8) upregulating mRNA-encoding $P450_{C17}$ (2), resulting in increased amounts of enzyme protein which can be detected by immunostaining (1,17).

In a recent paper it was suggested that androgen and estrogen production are not compartmentalized within the equine CL (1), which is in apparent contrast to findings in the human (19) and monkey (18) CL, which also produce ovarian estrogen via the Δ^4 steroidogenic pathway. In our previous studies we reported a clear difference in the location of $P450_{arom}$ and $P450_{C17}$ in follicles, with $P450_{arom}$ confined to the granulosa layer and $P450_{C17}$ present only in the theca layer (17,24). In the CL we found $P450_{arom}$ confined to large luteal cells (24), whereas another study (1) found $P450_{arom}$ in small luteal cells as well. Immunoreactive $P450_{C17}$ has been found in all luteal cells in the equine CL (1); however we failed to detect this enzyme in CL until after the onset of eCG secretion, and then only in selected luteal cell populations (17). In this latter study, immunoreactivity of the anti-human cytochrome $P450_{C17}$ antibody with equine $P450_{C17}$ had been confirmed by Western blotting (17). To clarify which cell populations within the equine CL contain $P450_{C17}$, we used a specific IgG fraction against $P450_{C17}$ raised in rabbits (kindly donated by Prof. J.I. Mason, Edinburgh) which had been purified from pig testes according to the method of Nakajin and Hall (15). Both the bioactivity and immunoreactivity of this antibody against equine $P450_{C17}$ had previously been demonstrated (14). For comparison, we used another antibody which had been employed in a previous study (17) and had also been shown to cross-react with equine $P450_{C17}$. Using these antibodies the distribution of $P450_{C17}$ in equine follicles and CL was determined by immunohistochemical techniques.

MATERIALS AND METHODS

Normally cyclic pony mares, weighing 250 to 400 kg, were used in this study. Ovulation was detected by transrectal ultrasonography and was designated as Day 0. Some mares were artificially inseminated for collection of CL from pregnant mares. Pregnancy was confirmed by transrectal ultrasonography.

Ovaries were obtained by hemiovariectomy, as previously described (23), during estrus (n=3), on Day 2 (n=2), and Days 8 to 10 (n=5) of the luteal phase in nonpregnant mares, and from pregnant mares on Day 20 (n=2) and on Days 40 to 80 (n=6) of pregnancy, after the onset of eCG secretion.

Immunohistochemistry

Pieces of tissue were embedded in OCT [a] and snap frozen in a dry ice/isopentane slurry. The tissue was then stored at -70°C until further processing. Tissue sections (7μm) were cut using a cryostat microtome and immunostained using an avidin-biotin complex method described previously (25). The primary antibody against $P450_{C17}$ (antibody A, Prof. Mason) was used at a dilution of 1:1,500. The chromagen used (3-amino-9-ethylcarbazole) produced a red reaction product and the sections were counterstained with haematoxylin. For comparison another primary antibody, raised in rabbits against human $P450_{C17}$ (antibody B, Dr. Waterman) was used at a dilution of 1:2,000 on frozen sections of the same tissue. Results using this latter antibody on paraffin-embedded equine tissue and a different immunostaining method have already been reported (17).

Negative control sections in which the first antibody was replaced by non-immune serum, and positive control sections of horse testis, were included with each batch of samples.

RESULTS

Using antibody A, immunoreactivity for $P450_{C17}$ was present in the theca interna of the preovulatory follicles. Most but not all theca cells stained positively. In places there was pale positive staining for $P450_{C17}$ in the granulosa cells (Figure 1a).

In the newly formed equine CL, the theca interna cells are seen as infoldings on the outer surface of the disrupted granulosa cell layer (12). These theca cells lose the characteristic polyhedral shape present in the preovulatory follicle and appear flattened and spindle shaped. Dense immunostaining for $P450_{C17}$ was present within the cytoplasm of the theca cells (Figure 1b).

In the mature CL, positively stained thecal-like cells were present in the trabeculae of the CL, which contain the vasculature (Figure 1c). In addition, these positively stained small cells were distributed in the parenchyma of the structure, lying between the large luteal cells (Figure 1d). The large cells contained only pale positive immunostaining, with a very few scattered cells staining heavily. This staining pattern remained the same in CL obtained from mares at Day 20 of pregnancy. In the CL collected after Day 40, occasional bands of staining were present which resembled the polyhedral-shaped theca cells seen in the preovulatory follicle (Figure 1e).

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[a] Miles Inc, Elkhart, IN.

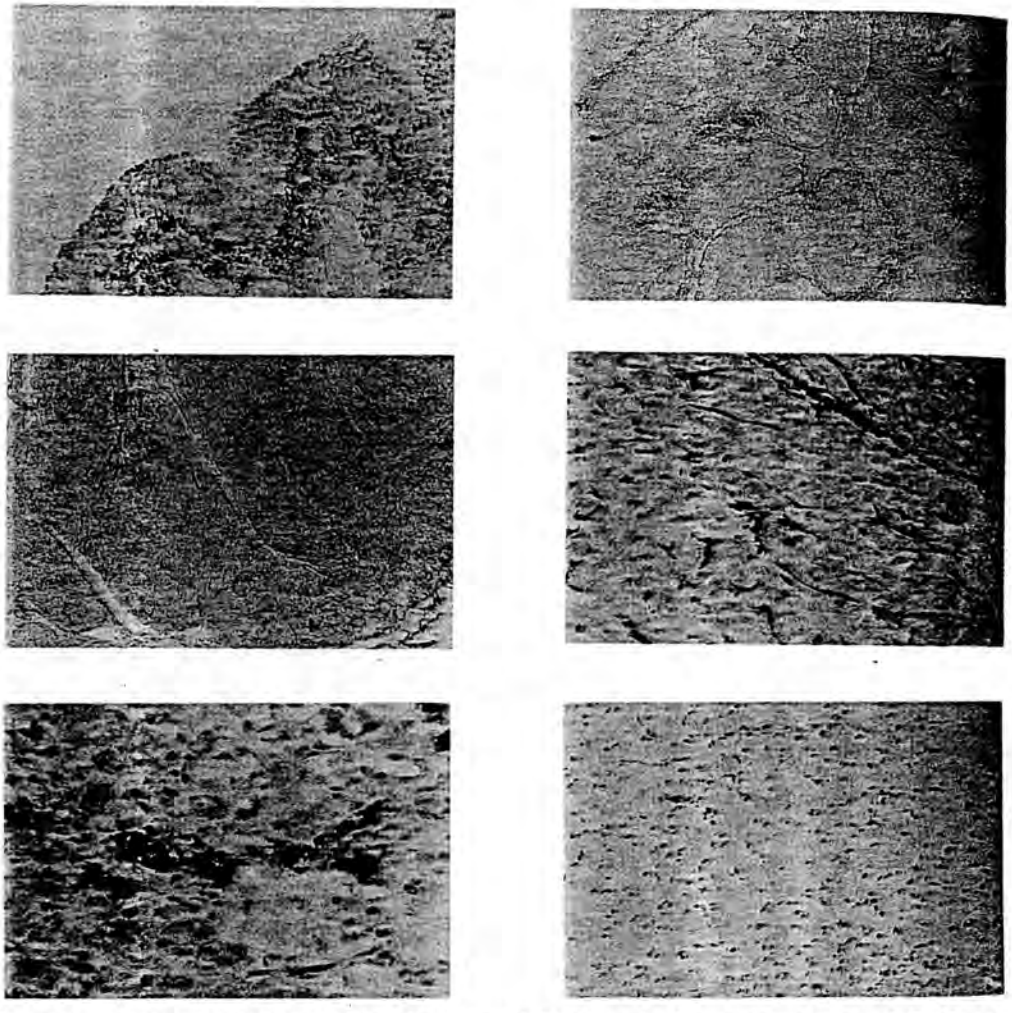

Figure 1. Immunohistochemical localization of $P450_{C17}$ in the equine ovary. a) $P450_{C17}$ staining in a preovulatory follicle. Specific immunostaining is present in the theca cells x 40. b) $P450_{C17}$ staining in a newly forming CL. Positive staining is present in cells on the outer surface of the disrupted granulosa cell layer x 4. c) $P450_{C17}$ staining in mid-luteal phase. Positively stained cells appear in the vascular tracts that converge in the trabeculae x 10. d) $P450_{C17}$ staining in mid-luteal phase. Heavily positive specific staining is present in the small fibroblast-like cells lying in between the large luteal cells x 20. e) $P450_{C17}$ staining in the CL from a Day 80 pregnant mare. A band of positively staining cells is present resembling the theca cell layer in the follicle x 20. f) Negative control section of CL from Day 8 of dioestrus in which the primary antibody was replaced by non-immune serum x 20.

Using antibody B, very similar staining patterns were observed to antibody A in the preovulatory follicle, the newly forming CL, and the CL obtained from mares after Day 40 of pregnancy. However no immunostaining was present in the mature CL from nonpregnant mares or from mares on Day 20 of pregnancy.

All control sections in which the primary antibody was omitted failed to show any immunostaining (Figure 1f). The interstitial cells in the horse testis stained positively for $P450_{C17}$, while the seminiferous tubules were negative.

DISCUSSION

We have clearly shown that small cells within the equine CL possess immunoreactivity for the steroidogenic enzyme $P450_{C17}$, providing direct evidence of the potential for these cells to contribute to steroidogenesis in the equine CL. Furthermore, in an earlier study we showed that dissociated small luteal cells produced progesterone in vitro (5). The difference in staining patterns between the 2 antibodies used in our present study illustrate possible differences in interpreting immunostaining results based on the antibody used. However, the patterns were the same in the follicle, the early CL and the CL after Day 35 of pregnancy, when it is likely that expression of $P450_{C17}$ is at its highest (1). It is possible that antibody B bound with lower affinity to equine $P450_{C17}$, which would explain the absence of immunoreactivity on Western Blot, and the lack of immunostaining in the mature CL during the cycle and before Day 40 of pregnancy (17). The marked difference of our immunostaining results from those of Albrecht and Daels (1), in which both large and small cells were found to stain heavily for immunoreactive $P450_{arom}$ and $P450_{C17}$ is less easy to explain. The antibody used by these workers was prepared in a manner similar to that of antibody A (J.I. Mason, personal communication), but presumably differences in immunostaining techniques, in tissue processing (paraffin-embedded sections versus frozen sections in our present study), or in antibody affinity resulted in the discrepancy between findings.

The intensely stained luteal cells in the mature equine CL were associated with blood vessels entering from the outer surface of the CL. An early study on formation of the equine CL after ovulation describes in some detail the fate of theca cells (12). These cells were reported to lose their polyhedral appearance apparent immediately before ovulation and invade the granulosa cells. The most marked accumulations of theca cells were seen in the trabeculae of the forming CL along with the thecal capillaries, but the theca cells were also observed between the luteinizing granulosa cells. The mature CL was described as having theca cells evenly distributed among the large luteal cells. Similar small cells were described in the equine CL by Van Niekerk et al. (22), but these authors claimed that the theca cells, which were originally present in the infoldings of the follicle wall, were replaced by hypertrophied fibroblasts in the maturing CL. Based on this

latter paper it is currently accepted that theca cells do not contribute to luteal tissue in the mare (11). However, the results of the present study would suggest that thecal-derived cells staining positively for $P450_{C17}$ are present between the large luteal cells in the equine CL.

It has been suggested but not yet confirmed that luteal cells retain functional differentiation from the progenitor follicular cells. In the human CL, luteinized granulosa cells maintain immunoreactivity for $P450_{arom}$, and luteinized theca cells immunostain positively for the presence of $P450_{C17}$ (19). A similar staining pattern was observed in the monkey CL (18). It is not likely that this can be taken as definite evidence of cellular origin, but a hallmark of the granulosa cell is sole expression of $P450_{arom}$ and that of the theca cell is expression of $P450_{C17}$ (9). Ultrastructural studies in the ovine CL have provided evidence that small luteal cells and fibroblasts of the CL derive from the theca interna (16). Furthermore, large cells isolated from early bovine CL are antigenically similar to granulosa cells, while small cells resemble theca cells (3), although it appears that later in the luteal phase some small cells may grow into large cells. In the present study, a few scattered large cells stained strongly positive, and all exhibited very pale positive immunostaining. This diversity of staining presumably reflects the heterogeneity of the large cell population, as we have described previously for $P450_{arom}$ (24), and as is recognized in other species (6). It has been suggested that cytodifferentiation may occur during luteinization that can cause the de novo expression of $P450_{C17}$ in granulosa-derived cells (9). Secondary CL, which form in pregnancy, may result from either ovulation or luteinization of follicles (10). The bands of positively staining cells in CL collected after Day 40 of pregnancy, which resembled follicular thecal cells, may have originated from luteinization rather than ovulation of follicles and represent luteinized thecal cells.

The fibroblastic appearance of many of the positively stained small cells in the present study is interesting in view of the close relationship between ovarian stromal and theca cells, with stromal cells differentiating into theca cells at the periphery of developing follicles (19). Furthermore, stromal cells surrounding follicles in the monkey ovary stained positively for $P450_{C17}$ (18). The relationship between stromal and theca cells in steroidogenesis needs further investigation. In the present study there was a clear difference in the location and morphology of cells expressing $P450_{C17}$ and those expressing $P450_{arom}$. The immunostaining characteristics were very similar to those of other species in which the CL produces estrogen (6,18,19). Earlier papers have confirmed the 2 cell theory for estrogen production in the equine follicle (1,21,24). The results of the present study strongly suggest that similar cooperation between small and large cells occurs in the equine CL, with small cells producing androgen after the onset of eCG secretion in sufficient quantity for the large cells to aromatize to estrogen. In view of these

results, the contribution of the theca cell to the equine CL should be more thoroughly investigated.

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Isolation and culture of glandular epithelial and stromal cells from the endometrium of mares

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Summary. Glandular epithelial and stromal cells were isolated from the endometrium of mares by collagenase digestion and were incubated on plastic for 7–9 days until the cells formed confluent monolayers. The cells differed in morphology: epithelial cells appeared polyhedral and stromal cells were spindle like. The monolayers were incubated in the presence and absence of oxytocin. Medium was removed from wells after 2, 8 and 24 h of incubation. Concentrations of prostaglandin F (PGF) in the medium increased significantly during this time. Glandular epithelial cells produced significantly more PGF than did stromal cells. Both types of cell responded significantly to oxytocin stimulation by increased secretion of PGF; the response of glandular epithelial cells tended to be greater than that of stromal cells. Secretion of PGF by cultured cells was not affected by cycle stage or pregnancy.

Keywords: endometrium; cell culture; prostaglandins; horse

Introduction

There is strong circumstantial evidence that prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) from the endometrium of nonpregnant mares is responsible for the demise of the corpus luteum near the end of dioestrus. In pregnant mares, the conceptus exerts an early inhibitory effect on the release of $PGF_{2\alpha}$ from the endometrium (Berglund *et al.*, 1982; Sharp *et al.*, 1989; Watson & Sertich, 1989). Oxytocin may be involved as an intermediary in release of $PGF_{2\alpha}$ in mares (Goff *et al.*, 1987) as it is in other species (Ginther *et al.*, 1967; Roberts *et al.*, 1975). It has been suggested that the response of the endometrium to oxytocin may differ in pregnant and nonpregnant mares (Goff *et al.*, 1987; Franklin *et al.*, 1989).

Although studies have been performed involving incubation of endometrial tissue in mares, little is known of the contribution of individual endometrial components to the events that occur during the oestrous cycle and at the time of maternal recognition of pregnancy. The endometrium is a complex tissue consisting of several types of cell. Investigation of the contribution of individual types of cell and the interactions between types of cell can be studied *in vitro* using primary cell cultures. In other species, epithelial and stromal cells from the endometrium have been shown to possess different characteristics with regard to growth in culture, sex steroid receptors (McCormack & Glasser, 1980; Varma *et al.*, 1982) and adenylate cyclase activity (Fortier *et al.*, 1987, 1988). In the present study, we isolated glandular epithelial cells and stromal cells from the mare endometrium and measured release of PGF by monolayers of these cells cultured in the presence and absence of oxytocin.

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Materials and Methods

Animals

Twelve mares of mixed breed weighing 450–550 kg were teased daily with a stallion. From the first day of oestrus, follicular development was monitored once a day by ovarian palpation and ultrasonography *per rectum* until signs of ovulation were detected. Day of ovulation was designated day 0. Endometrial biopsy samples ($n = 4$, approximately 1 g of tissue) were collected from the mares on day 3 of oestrus, days 10 or 14 of dioestrus, or day 14 of pregnancy. If samples were biopsied from a mare on more than one occasion, at least 12 days intervened between sample collections. Some of the mares were bred by artificial insemination during oestrus using semen from a fertile stallion and were confirmed pregnant by ultrasonic detection of an embryo before collection of endometrial biopsy samples on day 14.

Isolation of endometrial cells

Endometrial glands and stromal cells were isolated by a modification of the method described by Satyaswaroop *et al.* (1979) using reagents purchased from Sigma Chemical Co. (St Louis, MO, USA). The tissues were placed in cold Ham's F10 medium containing 2 mmol L-glutamine l^{-1} and 50 iu penicillin plus 50 μg streptomycin ml^{-1} until they arrived at the laboratory. The tissue was chopped into fragments of about 1 mm^3. Tissue fragments were disrupted further by gentle, repeated pipetting. The tissue was resuspended in Ham's F10 containing 30% heat treated newborn calf serum, 50 iu penicillin plus 50 μg streptomycin ml^{-1}, 2 mmol L-glutamine l^{-1}, 1·5 mg collagenase Type I (Sigma Chemical Co.) and 20 mmol Hepes l^{-1}. The suspensions were incubated at 39°C in a humidified atmosphere of 5% CO_2:95% air with agitation every 15 min. Digestion was periodically monitored by decanting a small volume of the suspension into a sterile tissue culture dish and visualizing the isolated glands under an inverted microscope. Digestion was allowed to continue until only an occasional stromal cell was left adhering to the glands (usually within 1–2 h). After incubation, red blood cells were removed by filtering the suspension through sterile gauze into a sterile polypropylene centrifuge tube. The cell suspension was then allowed to sediment for 10 min; during this time the glands settled on the bottom of the tube and the stromal cells remained in suspension. The supernatant containing the stromal cells was then removed and placed into a separate tube and centrifuged for 10 min at 200 *g*. Cells were resuspended in fresh medium (without collagenase) containing 10% newborn calf serum and any remaining glands were allowed to sediment. Sedimentation and washing were repeated twice. The supernatant containing mainly stromal cells was examined under an inverted microscope. If gland fragments were seen, the suspension was centrifuged at 100 *g* for 1 min to remove the gland fragments.

The gland pellet obtained from the first sedimentation step was resuspended in fresh medium containing 10% newborn calf serum and allowed to sediment. The supernatant was discarded and the process repeated 6–7 times to ensure a clean gland preparation.

Glands and stromal cells were each finally resuspended in medium containing 10% newborn calf serum. One millilitre of suspension was added to each of 18 wells of a 24-well tissue culture plate (Corning Glass Works, Corning, NY, USA) and incubated at 39°C in a humidified atmosphere of 5% CO_2:95% air. At 24 h, 1 ml of fresh medium was added and at 72–96 h the medium was changed. Cultures were incubated until a confluent monolayer was obtained (approximately 7–9 days), at which time the medium was removed. Fresh medium was added to control wells and medium containing oxytocin (1 μg) was added to replicate wells. The medium was removed from these wells after 2, 8 and 24 h (triplicate wells per time point), centrifuged at 3000 *g* for 10 min at 4°C and the supernatant stored in aliquots at −70°C.

After the medium was removed, 0·1% Triton X-100 was added to the wells and the cells were disrupted and detached from the plastic with the aid of a plastic scraper. The cell suspension was frozen at −20°C until assayed for protein concentrations using the method described by Lowry *et al.* (1951).

PGF assay

Medium was assayed directly for PGF using a previously validated technique (Watson & Sertich, 1989) with the exception that a different antibody (Sigma Chemical Co.) was used. Crossreactivity was less than 0·1% with all PGs tested except for $PGF_{1\alpha}$ (60% crossreactivity). Because of the high crossreactivity of the antiserum with $PGF_{1\alpha}$, results are reported as total immunoreactive PGF. Although the relative concentrations of the 'F' fraction of prostaglandins have not been measured in mare endometrium, in the rat uterus the predominant prostaglandin was $PGF_{2\alpha}$ with only trace amounts of $PGF_{1\alpha}$ (Fenwick *et al.*, 1977). It seems probable therefore that a major proportion of the PGF measured in this study was $PGF_{2\alpha}$. Limit of sensitivity of the assay was 0·1 ng per tube. Intra- and interassay coefficients of variation were 10·3% and 8·7%, respectively. The coefficient of correlation between amount recovered: amount added was 0·993 and regression analysis yielded an equation of $y = 1{\cdot}09x - 15{\cdot}65$.

Statistical analysis

Analysis of variance for repeated measures was used to evaluate the effects of stage of cycle, tissue type, treatment and time on concentrations of PGF. When indicated by the analysis of variance, Scheffe's test was used to make pairwise comparisons of means. A significance level of $P < 0{\cdot}05$ was used for all statistical tests.

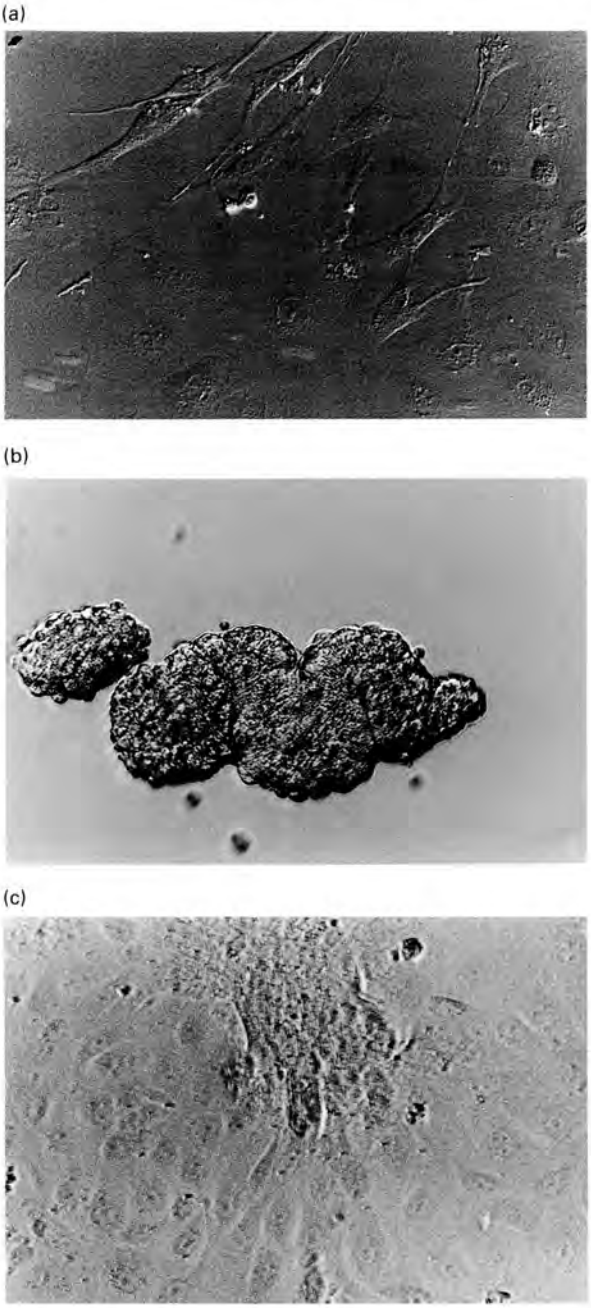

Fig. 1. Different stages of culture of isolated cells from mare endometrium: (a) stromal cells on day 2 of culture, (b) isolated glands and (c) gland collapsed with epithelial cells spreading and forming monolayer on day 2 of culture. Hoffman Modulation Contrast System, ×22.

Results

Examination by microscope throughout the culture period revealed that stromal and epithelial cells differed in morphology and cross-contamination of each cell type was judged to be <5%. More

than 95% of the stromal cells were viable at the time of plating based on trypan blue exclusion. Cell preparations consisted of either single cells or small clumps of cells. Stromal cells attached to the plastic by 24 h, and by day 2 cells were spreading and were spindle-shaped and fibroblast-like in appearance (Fig. 1a). After day 2, they rapidly spread to confluence.

Isolated glands were readily visualized under the microscope and were contaminated by a few stromal cells (Fig. 1b). By 24 h after plating, the glands had collapsed and a monolayer of cells was growing radially from each gland explant (Fig. 1c). Most of the gland cells within the monolayers were polyhedral, but some were elongated.

There was a significant ($P < 0{\cdot}01$) interaction between type of cell and time in concentrations of PGF measured in culture supernatant, indicating that PGF production over time was different for epithelial and stromal cells (Figs 2 and 3). Mean PGF production for the two types of cell was compared at 2, 8 and 24 h and the mean PGF concentration for the three periods was compared for each type of cell.

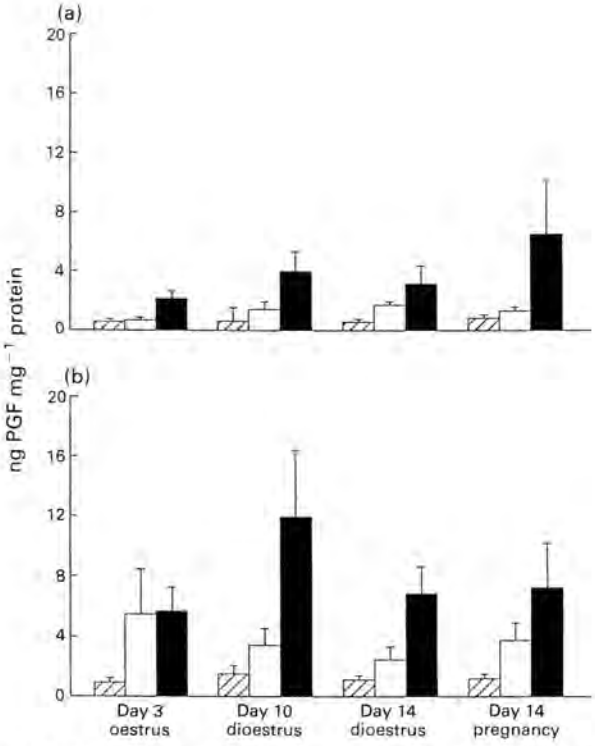

Fig. 2. Production of prostaglandin F (mean ± SEM) by a monolayer of mare endometrial glandular epithelial cells (a) in the absence and (b) in the presence of oxytocin. Supernatants were sampled at 2 (▨), 8 (□) and 24 (■) h after addition of fresh culture medium. Each point represents the mean of triplicate cell preparations from three to eight mares.

For glandular epithelial cells, there was a significant difference in mean PGF concentrations between 2 and 24 h and between 8 and 24 h. For stromal cells, there was a significant difference in mean PGF concentrations only between 2 and 24 h.

There was no difference in PGF production between glandular epithelial and stromal cells at either 2 or 8 h, but by 24 h, epithelial cells had produced significantly more PGF than stromal cells.

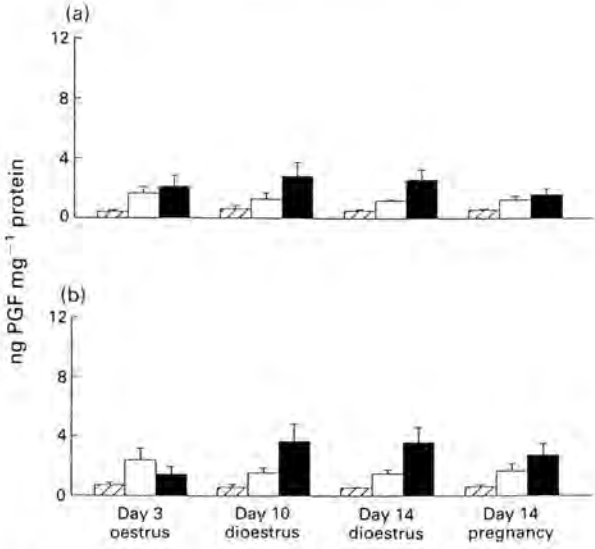

Fig. 3. Production of prostaglandin F (mean ± SEM) by a monolayer of stromal cells (a) in the absence and (b) in the presence of oxytocin. Supernatants were collected or sampled at 2 (▨), 8 (□) and 24 (■) h after addition of fresh culture medium. Each point represents the mean of triplicate cell preparations from three to eight mares.

Stage of cycle and pregnancy did not significantly affect production of PGF by glandular epithelial or stromal cells. Oxytocin treatment significantly ($P < 0{\cdot}01$) stimulated PGF secretion by both types of cell at all times, but the glandular epithelial cells tended ($P = 0{\cdot}053$) to respond more than stromal cells (Figs 2 and 3).

Discussion

Isolation and monolayer culture of endometrial stromal and epithelial cells has previously been reported in women (Satyaswaroop *et al.*, 1979; Casey *et al.*, 1985), cows (Fortier *et al.*, 1988), ewes (Cherny & Findlay, 1990) and rabbits (Ricketts *et al.*, 1983), but not in mares. Reported morphology was comparable across species, and similar to the results for endometrium of mares in our study. In other species, cell morphology has been shown to correlate well with immunohistological identification of cell markers and with identification of cells at an ultrastructural level (Satyaswaroop *et al.*, 1979; Varma *et al.*, 1982; Ricketts *et al.*, 1983; Cherny & Findlay, 1990). In our study, the stromal cell cultures did not appear to be contaminated with epithelial cells, presumably because they quickly overgrew any contaminating cells. By contrast, some cultures of glandular epithelial cells contained some fibroblast-like cells. Because glands always retained a few adherent stromal cells after the separation procedure, it is likely that these cells were the contaminating fibroblast-like cells. However, it has also been reported that epithelial cells tend to be pleomorphic after culture and some may appear spindle shaped resembling fibroblasts (Satyaswaroop *et al.*, 1979).

Previously, release of PGF by equine endometrium has been measured in harvested medium after incubation of endometrial tissue (Vernon *et al.*, 1981; Berglund *et al.*, 1982; Watson & Sertich, 1989) or tissue perfusion (Franklin *et al.*, 1989). In these studies, it was not possible to identify the cellular source of the PGF. By isolating types of cell, we showed that after incubation for 24 h, glandular epithelial cells had produced more PGF than had stromal cells. Isolated endometrial epithelial cells from the ewe and cow released most of the PGF from the endometrium (Fortier *et al.*, 1988; Cherny & Findlay, 1990), whereas in human endometrium it is not clear whether stromal

cells (Casey *et al.*, 1985; Gal *et al.*, 1982) or glandular epithelial cells (Schatz *et al.*, 1985; Smith & Kelly, 1988) are the major producers of PGF.

In the present study, concentrations of PGF significantly increased between 2 and 24 h after addition of fresh medium. Treatment of cell monolayers with oxytocin tended to produce a greater response in cultures of glandular epithelial cells than in stromal cells. The higher production of PGF by glandular epithelial cells along with the trend towards increased responsiveness to oxytocin may indicate that, in the mare, the epithelial cells are a more important source of PGF at luteolysis than are the stromal cells. The differences observed in production of PGF also help to confirm that two different cell populations were indeed being studied. Basal production of and release of PGF after oxytocin stimulation were not affected by pregnancy or stage of oestrous cycle when the tissue was collected. By contrast, short-term incubations of endometrium from mares produced greater concentrations of PGF during mid- to late dioestrus than at other stages of the cycle (Vernon *et al.*, 1981). In agreement with the findings of the present study, other workers have found no difference in basal production of PGF by endometrium from nonpregnant or early pregnant mares (Berglund *et al.*, 1982; Watson & Sertich, 1989; Franklin *et al.*, 1989). In our study, there was no statistical difference in response to oxytocin between cells from pregnant and nonpregnant mares 14 days after ovulation. Although there was no difference in mean concentrations of PGF between the control and oxytocin-treated glandular epithelial cells from pregnant mares after 24 h of incubation, mean PGF concentrations in control cultures were increased by one set of cultures which contained extremely high concentrations of PGF. Pairwise comparisons showed that there was a statistically significant increase in PGF concentrations in oxytocin-treated wells. Oxytocin is considered to be involved in luteolysis in the mare. Oxytocin binding by the endometrium of the nonpregnant mare increased during days 14–17 after ovulation (Stull & Evans, 1986). Administration of oxytocin to mares about the time of luteolysis stimulated release of $PGF_{2\alpha}$ from the endometrium (Betteridge *et al.*, 1985; Goff *et al.*, 1987); however, this response appeared to be inhibited in pregnant mares (Goff *et al.*, 1987). Similarly, *in vitro* treatment with oxytocin did not stimulate the release of PGF from perfused endometrium from pregnant mares (Franklin *et al.*, 1989). It is not clear why cultured endometrial cells from pregnant mares responded to treatment with oxytocin. Perhaps culturing the cells for 7 days altered the ability of the cells to respond to oxytocin, or perhaps lack of stromal–epithelial cell interactions modified the response. It is also possible that luminal and glandular epithelium may exhibit different patterns of secretion and, therefore, that the results of the present study cannot be compared with data from perfusion experiments or experiments using isolated luminal epithelium.

In conclusion, monolayers of glandular epithelial cells produced more PGF than stromal cells and PGF production was not affected by stage of cycle. Regardless of the pregnancy status of the mare at the time samples were obtained, the glandular epithelial and stromal cells responded to treatment with oxytocin. Further work with such monolayers will be necessary to investigate the manner in which substances produced by the conceptus interfere with synthesis of PGF by the endometrium of pregnant mares.

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Prostaglandin production by horse embryos and the effect of co-culture of embryos with endometrium from pregnant mares

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Summary. Embryos, endometrial biopsies, and uterine lavage fluid were collected from pregnant and non-pregnant mares 14 days after ovulation. Embryos were cultured for 20·5 h with and without endometrial tissue from pregnant mares, and endometrial tissue was cultured alone. Endometrial content of PGF tended to be higher ($P=0·06$) in non-pregnant than in pregnant mares, but the amount of PGF released from tissue during culture was similar for pregnant and non-pregnant mares. Lavage fluid from non-pregnant mares also tended ($P=0·08$) to contain higher concentrations of PGF. Co-incubation of embryos with endometrium from pregnant mares significantly ($P=0·01$) lowered concentrations of PGF in medium. Tissue concentrations and release of PGE-2 and 6-keto-PGF-1α were similar in endometrial samples from pregnant and non-pregnant mares and prostaglandin production was unaffected by the presence of an embryo during incubation. Horse embryos released all three prostaglandins during a 20·5-h incubation.

Keywords: mare; prostaglandins; pregnancy; embryo

Introduction

Maternal recognition of pregnancy by the mare occurs between Days 14 and 16 after ovulation (Sharp *et al.*, 1984). In the non-pregnant mare, prostaglandin (PG) F-2α appears to be the factor which terminates the life of the corpus luteum. At the time of luteolysis, increased concentrations of PGF have been measured in uterine venous blood (Douglas & Ginther, 1976), endometrium (Vernon *et al.*, 1981) and uterine lavage fluid (Zavy *et al.*, 1978). It has been shown that equivalent concentrations of PGF are released *in vitro* by endometrium from mares in early gestation and from non-pregnant mares, but co-culture of endometrium from pregnant mares with conceptus membranes significantly inhibits synthesis of PGF (Berglund *et al.*, 1982). This decreased synthesis of PGF suggests that, during early pregnancy, prostaglandins such as PGE-2 and PGI-2 may be synthesized in favour of PGF-2α.

In other domestic species, PGE-2 and PGI-2 have been shown to be luteotrophic or to antagonize the luteolytic effects of PGF-2α both *in vivo* and *in vitro* (Henderson *et al.*, 1977; Pratt *et al.*, 1977; Hueckstead & Weems, 1978; Milvae & Hansel, 1980; Fitz *et al.*, 1984). Intrauterine concentrations of PGE-2 increase during early pregnancy in ewes (Ellinwood *et al.*, 1979) and endometrial cells from pregnant ewes preferentially convert arachidonate (prostaglandin substrate) to PGE-2 (Marcus & Hackett, 1979).

Embryos also secrete prostaglandins which may contribute to pregnancy maintenance (Lewis *et al.*, 1982; Hyland *et al.*, 1982; Hwang *et al.*, 1988). Embryos of sheep and cattle secrete luteotrophic factors which stimulate progesterone production by luteal cells (Hickey & Hansel, 1987)

and prevent PGF-2α-induced regression of the CL (Vallet *et al.*, 1988). The concentrations and function of PGE-2 and PGI-2 secreted by the horse endometrium and embryo in early pregnancy have not been studied.

The purpose of the present study was to measure prostaglandins released by endometrium from pregnant and non-pregnant mares to determine whether substrate was being shunted preferentially to form prostaglandins other than PGF. The prostaglandin content of pregnant and non-pregnant mare endometrium at Day 14 was also assayed, as were uterine secretions. The presence of prostaglandins and luteotrophic substances in embryo culture medium was also determined.

Materials and Methods

Collection of endometria, uterine lavage fluid and embryos. Samples were collected from 14 light horse mares for a total of 25 cycles. All mares had Category I endometria by the criteria of Kenney (1978). Mares were teased daily and the ovaries examined *per rectum* by ultrasonography starting on the first day of oestrus. From the day the preovulatory follicle reached a diameter of 30 mm until ovulation, the mares were inseminated artificially daily or every 2nd day with semen from a fertile Standardbred stallion. Mares were checked by ultrasonography *per rectum* on Days 10–12 after ovulation for the presence of an embryonic vesicle and mares were classified as pregnant (N = 13) or non-pregnant (N = 9). Additional non-pregnant mares (N = 3) were included which had not been inseminated. On Day 14, ultrasonography was repeated to confirm the earlier diagnosis and uterine secretions were collected from some of the mares (6 pregnant, 8 non-pregnant) by transcervical uterine lavage of 50 ml phosphate-buffered saline using a method described previously (Watson *et al.*, 1987). The uteri of all mares, whether pregnant or non-pregnant, were then flushed with 2–3 litres of embryo collection medium (Dulbecco's phosphate-buffered saline without glucose, phenol red or pyruvate (GIBCO, Grand Island, NY, USA) and 1% new-born calf serum), as described by Douglas (1982), with the modification that a larger catheter was used (15 mm i.d.; Bivona, Gary, IN, USA). Ten singleton embryos and 3 sets of twin embryos were recovered. Embryos were transferred to Medium 199 (GIBCO) and the collection medium discarded. Three endometrial biopsies (Kenney, 1978) were then collected from each mare and placed in Medium 199. All samples were kept on ice until they reached the laboratory.

Uterine lavage fluid. Flushings were centrifuged at 2000 *g* for 10 min at 4°C and stored in aliquants at −70°C until assay.

Incubation procedure. Tissue and embryos were washed three times in incubation medium (Medium 199 with 50 units penicillin, 50 μg streptomycin, and 4·7 μl 7·5% bicarbonate solution per ml). The endometrium was chopped into approximately 2-mm fragments and weighed into 150 mg portions. One portion was frozen in a plastic snap top vial at −70°C until assayed for PG content. Other portions were incubated in 25 ml polyethylene tubes containing 10 ml incubation medium. Incubates were divided into the following groups: (1) endometrium (from 13 pregnant and 12 non-pregnant mares), (2) endometrium and indomethacin (N = 25, 4 μg/ml), (3) embryo (N = 9), (4) embryo and pregnant endometrium (N = 7).

Tissues were incubated in an humidified atmosphere at 5% CO_2: 95% air at 37°C. The incubation medium was changed after the cultures were allowed to equilibrate for 30 min. Cultures were removed after 20·5 h and centrifuged at 2000 *g*. Medium was stored in aliquants at −70°C until assay.

Luteal cell incubations. Two mares were ovariectomized on Day 12 after ovulation. Sedation and analgesia were achieved by i.v. administration of xylaxine and butorphanol. The ovaries were removed via the cranial vagina using an ecraseur: 3 corpora lutea were recovered. Single-cell suspensions were prepared by the method of Fitz *et al.* (1982) using a collagenase-digestion procedure. Cells were resuspended at a density of 5×10^5 per ml in 1 ml embryo incubation medium (pooled from 6 embryos) or in 1 ml incubation medium. Suspensions were incubated for 24 h in an atmosphere of 5% CO_2: 95% air at 37°C after which they were centrifuged at 2000 *g* and the medium was stored at −20°C until assayed for progesterone.

Progesterone radioimmunoassay. Progesterone was assayed directly in luteal cell culture medium using the method described by Watson & Hinrichs (1988). The main cross-reactivities of the antibody were 1·7% with 11-deoxycorticosterone, 2·4% with 11-deoxycortisol, 2% with 20α-dihydroprogesterone, and 1·3% with 5-β-pregnan-3,20-dione. The limit of detection of the assay was 25 pg/ml and the intra-assay coefficient of variation was 6%. All samples were included in one assay.

Prostaglandin radioimmunoassay. The assay used for PGF has been described previously (Watson *et al.*, 1987). Assays for PGE-2 and 6-keto-PGF-1α were performed using an iodinated tracer and reagents from New England Nuclear (N. Billerica, MA, USA). Significant cross-reactivities of antibodies were as follows: for PGF, 75% with PGF-1α and 1·5% with PGF-2β, for PGE-2, 3·7% with PGE-1; and for 6-keto-PGF-1α, 2·6% with PGF-2α, 1·9% with PGE-1, 1·4% with thromboxane B-2 and 1·1% with PGE-2. Prostaglandins were measured directly in medium and lavage without extraction. Various concentrations of prostaglandins were added to medium and lavage fluid and were quantitatively recovered in the assay. Correlations of amount added:amount recovered were 0·99 for all three assays.

Assay sensitivities were 0·25 ng PGF/ml, 2·5 pg PGE-2/ml and 20 pg 6-keto-PGF-1α/ml. Within-assay coefficients of variation for PGF, PGE-2 and 6-keto-PGF-1α were 6%, 3% and 4·3%, respectively. Samples for each prostaglandin were all analysed within one assay after establishing appropriate dilutions for each type of sample.

The extraction procedure for endometrium was as described by Olson *et al.* (1984). Briefly, the tissue was homogenized on ice in assay buffer containing 4 μg indomethacin/ml. Prostaglandins were extracted twice from the supernatant of the homogenate using ethyl ether. Recovery of radioactive tracer using this extraction procedure was 91·2 ± 5·07% (mean ± s.d.; $n = 10$). After drying down under nitrogen gas, the samples were suspended in phosphate-buffered saline and frozen at −70°C until assay.

Statistical analysis. Differences in prostaglandin concentrations were analysed using a one-way analysis of variance or a paired t test when appropriate. Differences were considered significant when $P < 0·05$.

Results

Culture medium from endometrial tissue incubated in the presence of indomethacin contained significantly ($P < 0·001$) lower concentrations (mean ± s.e.m.) of PGF (4·0 ± 2·2 ng/g), PGE-2 (26·0 ± 9·9 ng/g), and 6-keto-PGF-1α (27·3 ± 4·9 ng/g) than did tissue without indomethacin (Table 1). There were no significant differences in prostaglandin concentrations in endometrial tissue from pregnant and non-pregnant mares (Table 1), although concentrations of PGF tended to be lower in tissue from non-pregnant mares ($P = 0·06$). Similar concentrations of prostaglandins were released during culture of endometrial tissue from pregnant and non-pregnant mares. When embryos were co-incubated with tissue from pregnant mares, concentrations of PGF only were significantly lowered ($P = 0·01$). Lavage fluid from pregnant mares tended ($P = 0·08$) to contain lower concentrations of PGF than did lavage fluid from non-pregnant mares.

Table 1. Mean (± s.e.m.) quantities of prostaglandins in endometrium, uterine lavage fluid and released by endometrium during a 24-h incubation period from pregnant and non-pregnant mares

Sample	PGF	PGE-2	6-keto-PGF-1α	No.
Culture medium (ng/g)				
Non-pregnant endometrium	501 ± 160·4	142 ± 18·2	554 ± 96·0	12
Pregnant endometrium	422 ± 63·6[a]	139 ± 47·0	445 ± 73·3	13
Pregnant endometrium + embryo	178 ± 29·4[a]	87 ± 18·9	501 ± 143·6	7
Tissue (ng/g)				
Non-pregnant endometrium	510 ± 153·6[b]	244 ± 58·8	278 ± 86·9	12
Pregnant endometrium	173 ± 96·5[b]	176 ± 34·8	325 ± 62·7	13
Lavage (ng/ml)				
Non-pregnant	10 ± 4·3[c]	0·03 ± 0·01	0·33 ± 0·11	8
Pregnant	1·3 ± 0·9[c]	0·04 ± 0·02	0·24 ± 0·09	6

For values with similar superscripts: a, $P = 0·01$; b, $P = 0·06$; c, $P = 0·08$.

Embryos released prostaglandins into the medium during culture (Table 2). Concentrations tended to be higher after 20·5-h culture than in medium collected at 0·5 h.

Embryo culture medium had no luteotrophic influence on cultured luteal cells. Progesterone concentrations (mean ± s.e.m.) produced by cells incubated in medium were 17·7 ± 1·00 ng/ml, whereas concentrations in cells incubated in embryo culture medium were 18·7 ± 1·21 ng/ml.

Discussion

We have shown that 14-day-old horse embryos in culture release PGF, PGE-2 and PGI-2 (measured by the stable metabolite 6-keto-PGF-1α). Embryos of other species are known to produce prostaglandins (Hyland *et al.*, 1982; Lewis *et al.*, 1982; Hwang *et al.*, 1988) and are thought

Table 2. Quantity of prostaglandins (mean ± s.e.m.) in culture medium of 14-day-old embryos during 0·5-h and 20·5-h incubation periods

	Quantity (ng/embryo)	
Prostaglandin	0·5 h	20·5 h
PGF	1·2 ± 0·9	10·6 ± 3·4[a]
PGE-2	3·4 ± 0·7	8·4 ± 3·9[b]
6-keto-PGF-1α	1·7 ± 0·3	4·4 ± 0·8[c]

For values between 0·5 and 20·5 h: a, $P = 0·06$; b, $P = 0·08$; c, $P = 0·02$.

to make a significant contribution to the uterine milieu in maternal recognition of pregnancy. However, concentrations released by the embryos in the present study were very low compared with concentrations released by the endometrium. The embryos were not weighed and, therefore, it is not possible to make a direct comparison of prostaglandin production, but the relative area of endometrium in the mare's uterus compared with the size of the embryo suggests that the contribution of the embryo is negligible.

Nine of the mares which were included in the present study had been mated but were not pregnant 10–12 days after ovulation. Embryonic loss rate in fertile mares between Days 2–4 and 14 after ovulation has been shown to be 0–9% (Ball *et al.*, 1986, 1989) and most of these losses occur before the embryo reaches the uterus (Forde *et al.*, 1987). Therefore, it is unlikely that the uterine environment of these 9 mares had been influenced by the presence of an embryo.

In the present study, endometrium and uterine lavage fluid from pregnant mares contained less PGF than did those from non-pregnant mares but endometrium from pregnant mares was able to release similar amounts of PGF *in vitro* as endometrium from non-pregnant mares. These results are similar to those of previous studies (Vernon *et al.*, 1981; Berglund *et al.*, 1982). The transcervical method of collection of uterine lavage fluid causes release of endometrial PGF (Berglund *et al.*, 1982). However, it has been shown that significant differences exist between pregnant and non-pregnant mares regardless of the method of collection of the lavage fluid (Berglund *et al.*; 1982). It is probable that the decreased concentration of PGF measured in the present study after co-culture of the embryo with endometrial tissue from pregnant mares was due to inhibition of prostaglandin synthesis rather than increased metabolism as concentrations of PGF metabolite (15-keto-13,14-dihydro PGF-2α) in medium do not increase (Sharp *et al.*, 1984). The present study is the first published report on concentrations of PGE-2 and 6-keto-PGF-1α in pregnant and non-pregnant mares. No significant differences were detected in these prostaglandins in endometrial content, production, or uterine lavage fluid. Co-incubation of the embryo with endometrium did not significantly alter synthesis of PGE-2 or 6-keto-PGF-1α within the endometrium of pregnant mares. By contrast, it has been reported that PGE secretion by the maternal endometrium was inhibited by conceptus factors (Sharp *et al.*, 1989). Our hypothesis that prostaglandin substrate may be diverted from PGF to other prostaglandins is, therefore, incorrect.

In sheep and cattle, intrauterine infusion of secretory proteins from the early conceptus extends the length of dioestrus (Fincher *et al.*, 1986; Knickerbocker *et al.*, 1986; Vallet *et al.*, 1988). Measurement of prostaglandin concentrations revealed that these proteins inhibited endometrial synthesis of PGF but not of PGE-2 (Vallet *et al.*, 1988). Other workers have demonstrated the presence of an intracellular prostaglandin synthetase inhibitor within the endometrium of early pregnant cattle which inhibits formation of PGF but has a lesser effect on PGE-2 (Basu & Kindahl, 1987; Gross *et al.*, 1988). It seems likely that this endometrial prostaglandin synthetase inhibitor is linked with the conceptus inhibition of PGF synthesis. The changes in prostaglandin concentrations in the uterus of sheep and cows are similar to those measured within the mare endo-

metrium during early pregnancy. The inhibitory effect on PGF synthesis may, therefore, derive from conceptus proteins secreted into the culture medium. The molecular weight of the conceptus inhibitor substance in the mare is between 1000 and 6000 (Sharp *et al.*, 1989). The differential inhibitory effect on prostaglandin synthesis may indicate that enzyme steps which occur later than the cyclo-oxygenase enzyme system in the arachidonic acid cascade are being inhibited. It is also known that within the endometrium PGF derives primarily from epithelial cells and PGE-2 primarily from stromal cells (Grasso *et al.*, 1987), and that receptors are present for conceptus proteins at a cellular level (Godkin *et al.*, 1984), and so perhaps only cells secreting primarily PGF express the specific target receptors.

Although luteotrophic activity has been attributed to culture medium from 80% of 13–18-day cattle embryos (Hickey & Hansel, 1987), medium from horse embryos did not stimulate progesterone secretion by cultured horse luteal cells. Similarly, the major protein secreted by 14–16-day sheep conceptuses, ovine trophoblast protein 1 (no analagous protein has been identified in the mare; Zavy *et al.*, 1982), did not stimulate progesterone production by dispersed luteal cells (Godkin *et al.*, 1984). It is not known whether the difference in results reflects primarily a difference in culture techniques rather than a species difference.

The present experiment showed that horse embryos release prostaglandins *in vitro*. Co-incubation of embryos with endometrium from pregnant mares inhibited PGF synthesis, but not the synthesis of PGE-2 or 6-keto-PGF-1α. Further studies are needed to investigate the nature of this inhibition.

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DO MARES POSSESS AN INTRACELLULAR ENDOMETRIAL INHIBITOR OF PROSTAGLANDIN SYNTHESIS DURING EARLY PREGNANCY?

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ABSTRACT

Cytosol was prepared from endometrium collected from pregnant or nonpregnant mares 14 d after ovulation and was added to a prostaglandin-generating system (bovine fetal cotyledonary microsomes). Addition of endometrial cytosol from pregnant mares suppressed microsomal synthesis of prostaglandin F, but addition of endometrial cytosol from nonpregnant mares increased microsomal synthesis of prostaglandin F. The results suggest that mares possess an intracellular inhibitor of prostaglandin F synthesis during early pregnancy.

Key words: prostaglandin synthesis, pregnancy, mares

INTRODUCTION

There is strong circumstantial evidence that prostaglandin F-2α from the endometrium acts as the luteolysin in the mare. Concentrations of prostaglandin F in the uterine lumen (1) and uterine vein (2) and of the prostaglandin F metabolite, 13,14 dihydro-15-keto-prostaglandinF-2α (prostaglandin FM), in the peripheral circulation (3) increase at the time of luteolysis in the mare. In addition, administration of exogenous prostaglandin F-2α to mares results in premature termination of luteal function (2).

Concentrations of prostaglandin F in the uterine lumen (1) and uterine vein (2) and of prostaglandin FM in jugular blood (3) are significantly lower in early pregnant mares than in nonpregnant mares around the time of maternal recognition of pregnancy. There is evidence from in vitro studies that the conceptus exerts a

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direct effect on the endometrium to inhibit prostaglandin F production (4,5). This factor has recently been found to have a molecular weight between 1000 and 6000 (6). Vernon and co-workers (7) and Watson and Sertich (5) found that, in the absence of the conceptus, endometrium from pregnant mares released as much prostaglandin F as endometrium from nonpregnant mares. This might suggest that there are no endogenous inhibitory factors within the pregnant endometrium itself. However, recent work has shown in vitro that endometrium from early pregnant mares does not show an increased release of prostaglandin F after oxytocin stimulation (8). This suggests that the corpus luteum of early pregnancy is maintained because of endogenous inhibition of prostaglandin F synthesis or an inability to respond to stimulators of prostaglandin F release. In cattle, an endogenous inhibitor of prostaglandin F synthesis has been detected in the high-speed supernatant of homogenized bovine endometrium and in caruncular tissue during mid- to late gestation (9,10). Inhibition of prostaglandin F synthesis is much greater during early pregnancy than during the estrous cycle in cattle (10,11), and it has been proposed that this inhibition of prostaglandin F synthesis is induced in the maternal endometrium by conceptus secretory proteins, including bovine trophoblast protein-1 (12,13). Because inclusion of exogenous arachidonic acid does not counteract the inhibitory activity, it appears that bovine endometrial cytosol inhibits the cyclooxygenase enzyme rather than the earlier steps of arachidonate metabolism (10).

It is not known whether a mechanism exists for inhibition of prostaglandin F synthesis in the endometrium of the early pregnant mare at the time of maternal recognition of pregnancy. The present study used a preparation of bovine fetal cotyledonary tissue as a source of prostaglandin F-generating microsomes and investigated whether endometrial cytosol from mares was capable of inhibiting synthesis of prostaglandin F by the microsomes.

MATERIALS AND METHODS

Preparation of Microsomes (Prostaglandin-Generating System)

Bovine fetal cotyledonary tissue was collected within 1 h of calving and stored at -70°C until further processing. Microsomes were prepared by the procedure of Gross et al. (10). Briefly, tissue was homogenized, and the filtered supernatant was centrifuged at 800 g for 15 min at 4°C to remove nuclei and mitochondria. The supernatants were then centrifuged at 100,000 g for 60 min at 4°C to pellet the microsomes. The microsomes were resuspended in 0.1 M potassium phosphate (pH 7.5) and were stored in aliquots at -70°C.

Preparation of Equine Endometrium

Endometrial biopsy samples were collected from pregnant (n = 7) and nonpregnant (n = 3) horse mares 14 d after ovulation. Minced fragments of tissue were placed in Medium 199 (Sigma Chemical Co., St. Louis, MO) and incubated for 20.5 h at 39°C in an humidified atmosphere of 5% CO_2 : 95% air. The conditioned medium was harvested and used in another study and the tissue was stored at 70°C. Tissues from pregnant and nonpregnant mares were treated as described under preparation of microsomes. Because there was only a small amount of tissue available, the tissue of 5 of the pregnant mares was pooled. Tissues from other mares were processed separately. The high speed supernatant (cytosol) was stored in aliquots at -70°C.

Assay for Inhibition of Prostaglandin Synthesis

The technique described by Gross et al. (10) was used. Microsomes (equivalent of 500 mg tissue) were incubated in 0.1 M potassium phosphate buffer (pH 7.5) with 100 ug arachidonic acid (Sigma Chemical Co., St. Louis, MO) and with and without the high speed supernatant from the endometrium of pregnant or nonpregnant mares (250 mg tissue equivalent) for 1 h at 39°C in a shaking waterbath (2.6 ml total volume). Indomethacin (100 µg; a prostaglandin-synthesis inhibitor) was added to duplicate tubes as a positive control. The incubation was terminated by the addition of 0.25 ml ethanol. The tubes were centrifuged at 1500 g for 20 min at 4°C to pellet the precipitate and the supernatants were frozen at -70°C until being assayed for prostaglandin F.

Assay of Prostaglandin F

Incubation medium and cytosols were assayed for prostaglandin F using a direct radioimmunoassay previously described by Watson and Sertich (5) with the exception that a different antibody was used (Sigma Chemical Co., St. Louis, MO). Cross-reactivity of the antiserum was < 0.1% with all prostaglandins tested except for prostaglandin F-1α (60% cross-reactivity). Because of the high cross-reactivity of the antiserum with prostaglandin F-1α, results are quoted as total immunoreactive prostaglandin F. Limit of detection of the assay was 0.1 ng/tube. Intra- and inter-assay coefficients of variation were 10.3 and 8.7%, respectively. Concentrations of prostaglandin F present in cytosol added to incubations were subtracted from concentrations present in incubation medium.

Statistical Analysis

Effect of treatment on concentrations of prostaglandin F synthesized by microsomes was analyzed by a paired t-test. All means are quoted ± SEM.

RESULTS

Microsomes from bovine cotyledonary tissue synthesized large quantities of prostaglandin F. There were no significant differences in the cytosolic concentrations of prostaglandin F between the pregnant and nonpregnant endometrium. Figure 1 shows the concentrations of prostaglandin F generated by microsomes in the presence of endometrial cytosol from pregnant and nonpregnant mares. The cytosol preparation from the endometrium of pregnant mares abolished microsomal prostaglandin F synthesis. Concentrations measured were lower (32 ± 21.1%) than those originally added in the cytosol, but this was not significant. Concentrations also tended to be lower than those of the indomethacin control. In the presence of endometrial cytosol from nonpregnant mares, amounts of prostaglandin F synthesized by microsomes were increased, but not significantly (120 ± 14.2%), compared with that of the controls. Addition of indomethacin to microsomes significantly (P = 0.05) reduced synthesis of prostaglandin F.

DISCUSSION

Microsomes from bovine placental tissue synthesized prostaglandins and produced similar concentrations of prostaglandin F to those found in another study (10). In the present study, there was evidence of the presence of an intracellular prostaglandin F synthesis inhibitor in the endometrium of the pregnant mare. The degree of inhibition of prostaglandin F synthesis by endometrial cytosol of pregnant mares was greater than the degree of inhibition that occurred in the presence of

indomethacin. Because prostaglandin F synthesis was inhibited even in the presence of exogenous arachidonic acid, the in vivo inhibitory effect was probably mediated via the cyclooxygenase enzyme system rather than by interfering with intracellular arachidonate mobilization. It is not clear why cytosol from pregnant mare endometrium was apparently more effective at inhibiting synthesis of prostaglandin F than indomethacin. Preliminary studies had shown that 100 µg indomethacin induced a higher degree of inhibition of prostaglandin F synthesis than other concentrations tested. There was endogenous prostaglandin F present in the cytosol from the endometrium of pregnant mares, and the microsomes produced prostaglandin F during incubation. However, the post-incubation concentration of prostaglandin F measured in the medium was even lower than the pre-incubation concentration of prostaglandin F. It is possible that not only was production of prostaglandin F inhibited, but degradation of prostaglandin F also occurred. Prostaglandin is highly susceptible to enzymatic degradation at body temperature. Perhaps enzymes were present in the endometrial cytosol that metabolized the prostaglandin F during incubation.

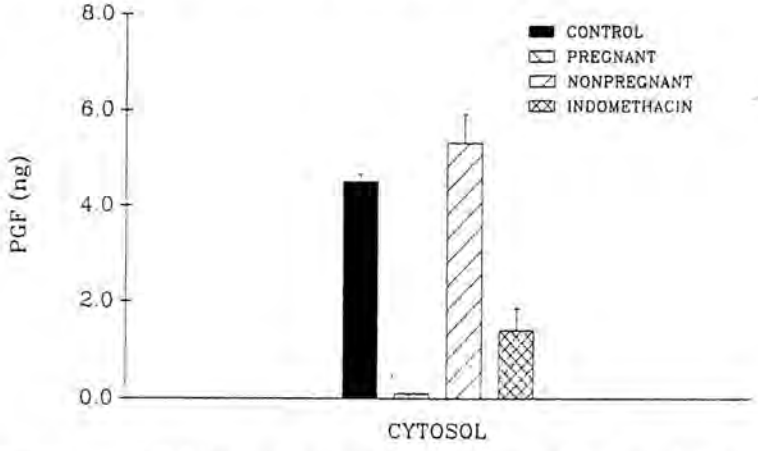

Figure 1. The effect of addition of endometrial cytosol from pregnant and nonpregnant mares on prostaglandin synthesis by microsomes. Cytosolic prostaglandin F concentrations were subtracted from prostaglandin F concentrations measured in incubation medium.

Endometrial cytosol from nonpregnant mares tended to increase prostaglandin F synthesis by microsomes, which may help to explain the increased synthesis of prostaglandin F by the nonpregnant mare endometrium at the time of luteolysis. By contrast, in cows exhibiting normal estrous cycles, some prostaglandin F inhibitory capacity was present even at the time of luteolysis (11).

It is possible, therefore, that the reduced secretion of prostaglandin F during early pregnancy in the mare is due to the presence of endogenous prostaglandin synthesis inhibitors within the endometrium. Release of prostaglandin by endometrium from pregnant and nonpregnant mares is similar. It is possible, therefore, that these inhibitors may be activated in the presence of the developing conceptus and may explain the reduced secretion of prostaglandin F during co-incubation of conceptus and endometrial tissue (5,6).

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Secretion of prostaglandins and progesterone by cells from corpora lutea of mares

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Summary. Corpora lutea (CL) were collected from mares during early (Day 4–5), mid- (Day 8–9), and late (Day 12–13) dioestrus. Dispersed cell suspensions were obtained by enzymic digestion of tissue. Two distinct luteal cell populations (large and small) were observed. The proportion of small luteal cells significantly increased as age of CL advanced.

Cells (2×10^6) from CL which were incubated for 24 h secreted prostaglandin (PG) F, PGE-2 and 6-keto-PGF-1α (the stable metabolite of prostacyclin). Higher concentrations of all PGs were produced by cells from CL at early dioestrus than from those at mid- or late dioestrus. The ratio of PGF:PGE-2 increased from 0·33 in CL of early dioestrus to 1·34 in CL of mid-dioestrus, whereas ratios of PGF:6-keto-PGF-1α remained relatively constant (~0·6). The ratio of PGE-2:6-keto-PGF-1α from CL decreased between early (3·27) and mid-dioestrus (0·43). Addition of LH, dbcAMP, or ionophore to cell cultures did not consistently affect secretion of progesterone or PGs by luteal cells. It is suggested that prostaglandins produced by luteal cells of mares may contribute to control of luteal function and that the changing ratios of prostaglandins may be more important in controlling the lifespan of the CL than absolute concentrations of each.

Keywords: mare; corpus luteum; prostaglandins; progesterone

Introduction

In domestic species, prostaglandins (PGs) have both luteolytic (Douglas & Ginther, 1972; Fitz *et al.*, 1984a) and luteotrophic (Hueckstead & Weems, 1978; Milvae & Hansel, 1983) roles. In the mare, unlike other species, the luteolytic effect of uterine PGF-2α apparently is not exerted via a local vascular pathway from the uterus (Ginther, 1974). Prostaglandin F-2α tends to have a very short half-life (seconds) once it enters the systemic circulation, and so only minute concentrations of uterine PGF-2α can reach the ovary. It is thought that the corpus luteum (CL) of the mare partly compensates by exhibiting an increased binding affinity for PGF-2α (F. A. Kimball, personal communication) compared with other species. However, it is also possible that tissues other than the uterus may contribute to PGF-2α synthesis at luteolysis. In cattle and primates it has been shown that luteal cells themselves secrete PGs (Patwardhan & Lanthier, 1980; Milvae & Hansel, 1983; Rodgers *et al.*, 1988). These studies suggest that local PGF may contribute to the regulation of luteolysis. Other PGs may also be produced by luteal cells which could influence the lifespan of the CL. PGE-2 and prostacyclin have been shown to be luteotrophic in other species (Hueckstead & Weems, 1978; Milvae & Hansel, 1983), but their effect on luteal cells of mares is not known.

There is no information regarding local production of PGs by ovarian tissue in the mare or whether these PGs could influence the lifespan of the CL. The present study measured production of PGF, PGE-2, 6-keto-PGF-1α (the stable metabolite of prostacyclin) and progesterone by dispersed equine luteal cells in culture. The effects of substances known in other species to affect

secretion of progesterone and PGs by luteal cells, e.g. indomethacin, luteinizing hormone (LH), calcium ionophore, and dibutyryl cyclic AMP (dbc AMP), were also evaluated.

Materials and Methods

Animals. Six horse mares (Thoroughbred and Standardbred) from 3 to 12 years of age were used. Throughout the spring and summer, all mares exhibited normal patterns of oestrous behaviour and ovulation. During the oestrous period under study, the mares' ovaries were examined daily by ultrasonography *per rectum* to determine day of ovulation, which was designated Day 0. The ovary containing the CL was removed at Day 4 or 5, Day 8 or 9, or Day 12 or 13 after ovulation. The remaining ovary was similarly removed at one of these time intervals after the next ovulation.

Surgery. Ovaries were removed using an ecraseur via an incision in the cranial vagina. Sedation and analgesia were achieved with intravenous administration of xylazine and butorphanol. Anaesthesia of the ovarian ligament was attained by local application of lignocaine before applying the ecraseur.

Preparation of luteal cells. After excision, ovaries were placed in Medium 199 (Gibco Laboratories, Grand Island, NY, USA) on ice and transported to the laboratory. The CL was dissected from the ovary and the capsule was removed. The tissue was then minced and weighed. Dispersed cell suspensions were prepared by collagenase digestion: 5 g luteal tissue were added to 10 ml Hanks balanced salt solution (calcium- and magnesium-free; Gibco Laboratories) supplemented with 7·6 mg collagenase, 2 mg deoxyribonuclease, 20 mM-Hepes buffer, 500 units penicillin, 500 μg streptomycin, and 1% bovine serum albumin. All additives were purchased from Sigma Chemical Company (St Louis, MO, USA). Dissociation of cells was achieved by incubation for 45 min at 37°C in a shaking water bath. The mixture was then centrifuged for 10 min at 125 *g*. The supernatant was discarded and the incubation repeated with fresh medium containing collagenase for a further 45 min. After further centrifugation, the cells were resuspended in a small volume of Medium 199. Clumps of cells were dispersed by repeated gentle aspiration into a sterile syringe. After straining the mixture through a gauze filter, the cells were washed twice in Medium 199 and then resuspended in culture medium (Medium 199 containing 50 units penicillin, 50 μg streptomycin, 10% heat-inactivated newborn calf serum, and 4·7 μl 7·5% bicarbonate per ml). By this stage, single cell suspensions were obtained apart from a few of the small luteal cells which tended to remain in small sheets of tissue. Similar degrees of dissociation were obtained for tissue from mares at all stages of dioestrus. Luteal cells were counted using a haemocytometer and suspended in medium at 5×10^5/ml. A smear of each cell suspension was prepared for histological examination using a cytocentrifuge and stained with Diff-Quik (Baxter Scientific, McGraw Park, IL, USA) to differentiate cells.

Luteal cell incubations. Incubations were performed at 37°C in a humidified atmosphere of 5% CO_2:95% air. Luteal cells (4 ml) were added to 60-mm sterile plastic tissue culture dishes (Becton Dickinson, Lincoln Park, NJ, USA) and incubated for 24 h. After incubation, the suspensions were centrifuged at 2000 *g* for 10 min to pellet non-adherent luteal cells and the supernatant was stored at −20°C until assay. To check whether progesterone was being synthesized by cells in culture rather than being passively released, 1 ml cells from each of 5 CL were frozen before incubation as well as after a 2-h incubation. The cells were lysed by freezing and thawing and cell contents and medium were assayed.

Treatments. The following treatments were applied in a volume of 0·1 ml to triplicate culture dishes. Concentrations quoted are the final concentrations in medium: dbcAMP (1 mmol/l; Sigma Chemical Co.), calcium ionophore (40 μg/ml; Calbiochem, San Diego, CA, USA), indomethacin (4 μg/ml; Sigma Chemical Co.), and horse LH (100 ng/ml).

Progesterone assay. The assay has been described previously (Watson & Hinrichs, 1988). The assay was performed directly in centrifuged culture supernatant without extraction. The samples containing cells (the 0 and 2-h cultures) were extracted twice in petroleum ether before assay. Recovery of progesterone after extraction was 95%. The main cross-reactivities (>1·0%) of the antiserum were with progesterone 100%, 11-deoxycorticosterone 1·7%, 11-deoxycortisol 2·4%, 20α-dihydroprogesterone 2%, and 5β-pregnan-3,20-dione 1·3%. The limit of detection of the assay was 25 pg/ml. Within- and between-assay coefficients of variation were 6% and 9%, respectively.

PGF assay. Concentrations were measured directly in culture medium. The method has been described previously (Watson *et al.*, 1987). The main cross-reactivities (>1·0%) of the antiserum were PGF-2α 100%, PGF-1α 75%, and PGF-2β 1·5%. Standard curves prepared in assay buffer and culture medium were parallel. Recovery of PGF was 102 ± 6·8% (mean ± s.d.). The coefficient of correlation of added:recovered PG was 0·99. Limit of detection of the assay was 0·25 ng/ml. Within-assay coefficient of variation was 6%. All samples were included in one assay.

PGE-2 assay. Reagents were purchased from New England Nuclear (North Billerica, MA, USA). An iodinated tracer was used. Samples were assayed directly without extraction at a dilution of 1:10. Main cross-reactivities (>1·0%) of the antibody were with PGE-2 100% and PGE-1 3·7%. Standard curves prepared in assay buffer and culture medium were parallel, with a coefficient of correlation of 0·99 between added and recovered PGE-2. Recovery of PGE-2 was 102 ± 8·2% (mean ± s.d.). The limit of detection of the assay was 2·5 pg/ml. Within-assay coefficient of variation was 3% and all samples were included in one assay.

6-Keto-PGF-1α assay. Reagents were purchased from New England Nuclear. Samples were assayed directly at a dilution of 1:10. Major cross-reactivities (> 1·0%) of the antibody were with 6-keto-PGF-1α 100%, PGF-2α 2·6%, PGE-1 1·9%, TXB-2 1·4% and PGE-2 1·1%. Standard curves prepared in assay buffer and culture medium were parallel. The correlation coefficient for added:recovered 6-keto-PGF-1α was 0·99. The limit of detection of the assay was 20 pg/ml. Recovery of 6-keto-PGF-1α was 85 ± 15·1% (mean ± s.d.). Within-assay coefficient of variation was 4·3%. All samples were performed in the same assay.

Statistical analysis. Differences in hormone concentrations or in luteal cell numbers were analysed using analysis of variance. Correlations were tested using Pearson's test. Levels of significance of $P < 0·05$ were considered significant.

Results

CL collected during early, mid- or late dioestrus were similar in weight (Table 1). The number of cells isolated from these CL did not vary significantly. Isolated cells consisted mainly of three distinct cell types: (1) large luteal cells (20–65 μm; mean ± s.e.m. 42 ± 1·8 μm) which were large, polyhedral cells with vacuolated cytoplasm and a single round or oval eccentric pale nucleus containing one or more nucleoli; (2) small luteal cells (7–18 μm; 11·1 ± 0·4 μm) which were smaller, often elongated with irregular dark-staining nuclei; and (3) a few spindle-shaped endothelial cells. Moderate contamination with red blood cells was common as were small numbers of leucocytes. The proportion of large luteal cells decreased significantly between mid- and late dioestrus (Table 1), but actual numbers did not change significantly ($2·1 \times 10^7$ in mid-dioestrus; $6·0 \times 10^7$ in late dioestrus).

Table 1. Weights, cell numbers and ratio of large to small cells isolated from CL of mares

	Age of corpora lutea		
	4–5 days	8–9 days	12–13 days
No. of mares	4	4	4
Weight of CL (g)	6·7 ± 0·6	5·6 ± 0·2	5·5 ± 1·0
No. of luteal cells per CL ($\times 10^{-7}$)	5·7 ± 1·5	5·5 ± 2·0	24·5 ± 12·7
No. of large:small luteal cells (%)	46 ± 4·7[a]	38·5 ± 0·9[b]	24·3 ± 2·0[a,b]

Values are mean ± s.e.m.
Values with similar superscripts differ significantly: a, $P < 0·01$; b, $P < 0·001$.

Over a 2-h incubation period, there was a 53 ± 3·9% (s.e.m.) increase in progesterone content of cells plus medium. By 24 h, there was a 2–4-fold increase in progesterone concentrations compared with concentrations at 2 h. In one experiment, plating efficiency of cells was 71% as determined by counting cells present in culture medium aspirated after 24 h. Before plating, 80% of cells were viable (judged by trypan blue exclusion). After 24 h, 77% of the cells which had not adhered to the plate were viable.

Over a 24-h incubation period, progesterone production by cells did not vary significantly with stage of cycle (532 ± 252 ng on Day 4–5; 1124 ± 307 ng on Day 8–9; 258 ± 76·2 ng on Day 12–13) or after addition of LH, indomethacin, dbcAMP or calcium ionophore (data not shown).

Luteal cells from CL collected early in dioestrus produced significantly more PGs ($P < 0·01$) than did cells from mid-dioestrus CL and more PGF ($P < 0·01$), PGE-2 ($P < 0·01$), and 6-keto-PGF-1α ($P < 0·05$) than did cells from late CL (Fig. 1). There were no significant differences between concentrations of PGF and 6-keto-PGF-1α produced by luteal cells collected in mid- or late dioestrus, but PGE-2 increased significantly ($P < 0·05$) between mid- and late dioestrus. There were significant ($P < 0·01$) positive correlations between concentrations of the PGs produced by luteal cells. Cultures to which indomethacin was added generally contained lower concentrations of PGs than did control cultures. Luteal cells from CL at mid-dioestrus tended to synthesize little

PGF, and there was no significant difference in PGE-2 concentrations in cultures with and without indomethacin. Neither LH nor dbcAMP significantly affected PG production, but ionophore decreased release of PGE-2 and 6-keto-PGF-1α from early CL.

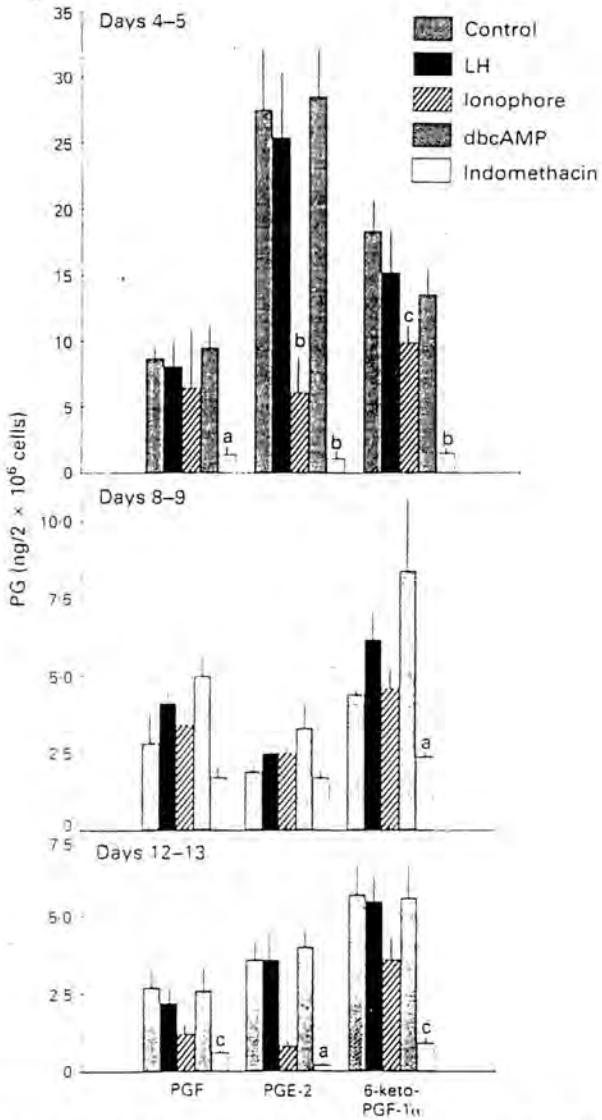

Fig. 1. Release of prostaglandins from luteal cells of mares during early, mid- and late dioestrus after treatment with LH (100 ng/ml), ionophore (40 μg/ml), dbcAMP (1 mmol/l) or indomethacin (4 μg/ml). Values are mean ± s.e.m. for 4 CL per stage of cycle. Values differing significantly from controls: a, $P < 0{\cdot}001$; b, $P < 0{\cdot}01$; c, $P < 0{\cdot}05$.

The ratio of PGF:PGE-2 released by luteal cells increased as the cycle advanced (Table 2). By contrast, the ratio of PGF to 6-keto-PGF-1α remained relatively constant in cultures of luteal cells collected from early through late dioestrus. The ratio of PGE-2:6-keto-PGF-1α released by luteal cells decreased between early and mid- and late dioestrus.

Table 2. Ratios (mean ± s.e.m.) of prostaglandins released by luteal cells of mares during a 24-h incubation period

	Age of corpora lutea		
	4–5 days	8–9 days	12–13 days
PGF:PGE-2	$0{\cdot}33 \pm 0{\cdot}04^{a,b}$	$1{\cdot}34 \pm 0{\cdot}24^{a}$	$0{\cdot}74 \pm 0{\cdot}18^{b}$
PGF:6-keto-PGF-1α	$0{\cdot}52 \pm 0{\cdot}68$	$0{\cdot}61 \pm 0{\cdot}18$	$0{\cdot}72 \pm 0{\cdot}31$
PGE-2:6-keto-PGF-1α	$3{\cdot}27 \pm 1{\cdot}18^{c,d}$	$0{\cdot}43 \pm 0{\cdot}05^{c}$	$0{\cdot}82 \pm 0{\cdot}26^{d}$

For differences between cycle stage: a, $P < 0{\cdot}001$; b, $P < 0{\cdot}05$; c, $P < 0{\cdot}01$; d, $P < 0{\cdot}05$.

Discussion

In this study, luteal cells from the CL of mares in early, mid- and late dioestrus released PGs, and concentrations decreased as the age of the CL increased. A similar pattern of PG release has been reported using luteal cells collected from cattle at different stages of dioestrus (Rodgers *et al.*, 1988), but in the horse, PGE-2 tended to be the predominant PG released by the early CL rather than 6-keto-PGF-1α. Addition of indomethacin (a known inhibitor of prostaglandin synthesis) to cultures lowered concentrations of PGs in culture medium, indicating that PGs were actively being synthesized by the luteal cells during incubation. Red blood cells reportedly do not secrete PGs (Higgs, 1982). Therefore, the contaminating red blood cells in luteal cell cultures probably did not contribute to concentrations of PGs measured in medium. High concentrations of PGE-2 and 6-keto-PGF-1α were released by luteal cells early in the cycle. In the cow, it is thought that prostacyclin may be luteotrophic early in the development of the CL specifically by controlling proliferation of blood vessels (Milvae & Hansel, 1983). The decreased synthesis of PGF by cells cultured from late CL of mares suggests that locally produced PGF is not primarily responsible for luteolysis in this species. However, the ratio of PGF:PGE-2 released by luteal cells during culture increased as the cycle advanced. In other species, PGE-2 is thought to be luteotrophic (Hueckstead & Weems, 1978; Fitz *et al.*, 1984b) and it has been postulated that an increase in the ratio of luteolytic to luteotrophic concentrations of PGs during the later stages of the cycle rather than concentrations of individual PGs may be of significance in luteolysis (Balmaceda *et al.*, 1979; Patwardhan & Lanthier, 1980; Milvae & Hansel, 1983; Rodgers *et al.*, 1988). It is possible that a similar mechanism operates in the mare. However, administration of PGE to cyclic mares did not prolong the lifespan of the CL (see Sharp *et al.*, 1989).

Both calcium ionophore and dbcAMP stimulate PG synthesis by luteal cells of other species (Johnson *et al.*, 1988; Rodgers *et al.*, 1988). In the present study, neither was stimulatory when added to horse luteal cells, and inophore frequently tended to decrease PG synthesis.

Horse LH has been shown to increase production of progesterone by luteal cells of mares (Kelly *et al.*, 1988b), and in other species dbcAMP acted like LH in stimulating progesterone production by cultured luteal cells (Godkin *et al.*, 1977; O'Shaughnessey & Wathes, 1985). In our study, progesterone production by horse luteal cells was not stimulated by ionophore, dbcAMP or LH (data not shown). It is possible that differences between the present and previous studies may be a function of the culture conditions. For example, it has been shown that LH does not stimulate progesterone production by sheep and cattle luteal cells when they are cultured in a medium containing serum (Pate & Condon, 1982; Hoyer *et al.*, 1988). We suggest that the presence of LH in the serum may already be maximally stimulating the luteal cells. Alternatively, numbers of LH receptors could be decreased during culture under certain conditions.

In the present study, addition of indomethacin to luteal cell cultures did not affect progesterone production, showing that PGs did not influence progesterone synthesis under these culture conditions. Similar results have been reported for luteal cells of cattle (Milvae & Hansel, 1983; Pate & Condon, 1984).

In the present study, cells were not stained for 3β-hydroxysteroid dehydrogenase activity and, therefore, the number of steroidogenic cells is not known. More than 80% of luteal cells isolated from mid-cycle sheep CL are non-steroidogenic (Fitz *et al.*, 1982). In the present study, the increase in progesterone concentrations measured in cells plus medium 2 h after the start of incubation, compared with before incubation, shows that progesterone was being synthesized by the luteal cells. No significant differences in progesterone production by luteal cells were detected at different stages of the cycle, although concentrations tended to decrease later in the cycle.

It is likely that the numbers of luteal cells harvested by dispersion are considerably lower than the actual cell content of the CL. Work performed with sheep and cow CL has shown that cell dissociation results in loss of cells with possibly selective loss of certain cell types (Rodgers *et al.*, 1984; O'Shea *et al.*, 1989). The ratio of small to large luteal cells reported in this study agrees, however, with morphometric studies on mare luteal tissue (Van Niekerk *et al.*, 1975), and the proportion of small to large luteal cells increased as the cycle progressed. In other species, the two populations of luteal cells have distinct functional properties with regard to progesterone synthesis and sensitivity to LH and PGs (Koos & Hansel, 1981; Fitz *et al.*, 1982, 1984a, b; O'Shea, 1987; Alila *et al.*, 1988a, b). In other species, the proportion of small to large luteal cells decreases as the cycle advances (Fitz *et al.*, 1981; Niswender *et al.*, 1985) and it is thought that these cell changes facilitate control of the lifespan of the CL, i.e. increased numbers of large cells in late dioestrus which bear the majority of the receptors for PGF-2α, thereby increasing the sensitivity of the CL to the action of PGF-2α. There is no information regarding functional properties of small and large luteal cells in the mare.

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Suppression of Lymphocyte Reactivity by Culture Supernatant from Horse Embryos and Endometrium[1]

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ABSTRACT

The mechanisms that permit maternal tolerance of the conceptus allograft during early pregnancy in the mare have not been investigated. Embryos and endometria were collected from mares 14 days after ovulation and cultured for 20.5 h. The effect of addition of culture supernatant on incorporation of [^{3}H]thymidine by equine peripheral blood lymphocytes was studied. Culture supernatant from endometrium of nonpregnant mares did not affect lymphocyte blastogenesis, but supernatant from both embryos and endometrium of pregnant mares reduced concanavalin A (Con A)- and phytohemagglutinin-induced blastogenesis. Five of six cultures performed in the present of indomethacin did not contain immunosuppressive factors. The suppressive effect on Con A-induced blastogenesis was eliminated by charcoal treatment of the supernatants and reduced by treatment with trypsin or heat. Blastogenesis of bovine lymphocytes was inhibited by culture supernatant of endometrium from pregnant mares, but not by embryo supernatant. Preincubation of blood lymphocytes with supernatants from endometrium of pregnant mares enhanced subsequent incorporation of [^{3}H]thymidine by lymphocytes. A 24-h delay in addition of embryo culture supernatants significantly reduced the degree of immunosuppression. These results suggest that probably more than one substance interacts with the lymphocyte cultures and the observed blastogenesis reflects the end result of the interaction between suppressive and stimulating factors. The lymphocyte inhibitory effect evident in supernatants from embryos and endometrium from pregnant mares may be important in local immunosuppression and maternal acceptance of pregnancy.

INTRODUCTION

The uterus of the mare is capable of mounting an effective immune response after introduction of antigen (Widders et al., 1985; Watson et al., 1987). Developing conceptuses express major histocompatibility complex (MHC) Class I antigens that might elicit an immune response and rejection of the conceptus (Goldbard et al., 1985). Antibodies directed against the zona pellucida have been detected in some infertile mares (Liu and Shivers, 1982) and, in mice, antibody molecules can penetrate the zona pellucida (Cozad and Warner, 1981). The presence of the capsule in equine embryos does not appear to mask embryonic antigens (White et al., 1988). Studies in mice have shown that early durring embryonic development the zona pellucida protects the embryo from cytotoxic T lymphocytes (Ewoldsen et al., 1987), but in the mare the zona pellucida starts disappearing around Day 8 of pregnancy (Ginther, 1979). It has been shown that at the time of development of the endometrial cups, a population of suppressor cells is present (Kydd and Allen, 1986) that may inhibit the maternal immune response at this stage of pregnancy. There is no information concerning acceptance of the equine conceptus at the time of maternal recognition of pregnancy or explaining why normal function of effector lymphocytes is blocked in the mare at this time.

In lagomorphs (Dey et al., 1981), ruminants (Segerson, 1981; Segerson et al., 1984; Fisher et al., 1985), and pigs (Murray et al., 1987), factors have been identified in uterine secretions and embryo culture supernatant collected during early pregnancy that suppress blastogenesis of peripheral blood lymphocytes in vitro. The lymphocyte inhibitory factors have been tentatively identified in the sheep and pig as a high molecular-weight acidic glycoprotein (Murray et al., 1987), in the cow as a small peptide (French and Northey, 1983; Fisher et al., 1985), and in the mouse as prostaglandin E_2 (PGE_2) (Tawfik et al., 1986). In uterine secretions

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from ewes (Segerson, 1981) and in culture medium from bovine embryos (Croy et al., 1988), lymphocyte inhibitory activity has been shown to be greater at Day 14 of pregnancy than at earlier stages.

The present study investigated whether supernatant from short-term cultures of endometrium from pregnant and nonpregnant mares and from embryos contained substances that influenced mitogen-induced proliferation of equine and bovine lymphocytes.

MATERIALS AND METHODS

Collection of Embryos and Endometrium

A total of 11 light horse mares were used. Mares were inseminated while in estrus with semen collected from a fertile stallion. Day of ovulation was determined by ultrasonography and was designated Day 0. Pregnancy was diagnosed by ultrasonographic detection of an embryonic vesicle between Days 10 and 12. Six of the eleven mares were diagnosed as pregnant. On Day 14 after ovulation, the uterus of each mare was flushed with 300 ml of Dulbecco's phosphate-buffered saline (PBS; GIBCO, Grand Island, NY) containing 1% fetal calf serum. The embryo collection procedure was as described by Douglas (1982), using a catheter with an internal diameter of 15 mm (Bivona, Gary, IN). Immediately after flushing, 3 endometrial biopsy samples were collected from the base of the uterine horns of each mare using the technique described by Kenney (1978). Biopsy samples and embryos were stored in ice-cold Medium 199 (GIBCO) until they were transported to the laboratory.

Culture of Embryos and Endometrium

Tissues were cultured as described previously (Watson and Sertich, 1989). Briefly, endometrium was washed in Medium 199 and then minced finely. Two 150-mg aliquots were cultured in sterile 50-ml polypropylene tubes (Falcon, Becton Dickinson, Lincoln Park, NJ) containing 10 ml Medium 199 with 47 μl 7.5% sodium bicarbonate and 500 units penicillin plus 500 μg streptomycin. Embryos were also rinsed and cultured intact in 10 ml medium in the presence or absence of 4 μg indomethacin/ml. Tissues were incubated in a humidified atmosphere of 5% CO_2:95% air for 20.5 h at 37°C. At the end of incubation, culture medium was centrifuged at 2000 × *g* for 10 min, and the supernatant was stored in aliquots at −70°C.

Lymphocyte Blastogenesis Assay

Blood was collected from healthy horses or cows into evacuated tubes containing ethylenediaminetetraacetic acid. The tubes were centrifuged at 2000 × *g* for 10 min, and the buffy coats were collected. The buffy coats were washed twice in PBS (pH 7.4) and resuspended in 10 ml PBS. Cells (5 ml) were layered on top of Histopaque (5 ml; Sigma Chemical Co., St. Louis, MO) in a 15-ml conical centrifuge tube. Tubes were centrifuged at 400 × *g* for 30 min at 24°C. The cells at the interface (>95% mononuclear cells) were removed, washed three times in PBS, resuspended in complete medium (RPMI-1640, GIBCO, containing 10% fetal calf serum, 100 units penicillin plus 100 μg streptomycin/ml, 2 mM L-glutamine and 10^{-5}M 2-mercaptoethanol), and counted with a hemocytometer. Viability was assessed by exclusion of trypan blue dye. With this procedure, more than 95% of the isolated mononuclear cells were viable. Cells were diluted to 4×10^6/ml.

The blastogenesis assay was performed in polystyrene 96-well flat-bottomed microtiter plates (Corning, New York, NY). Cells (50 μl, 2×10^5) were added to each well followed by culture supernatant (100 μl). Each culture supernatant was added to quadruplicate wells. Concanavalin A (Con A, 50 μl, 20 μg/ml, Sigma Chemical Co.) and phytohemagglutinin A (PHA, 50 μl, 12.5 μg/ml, Sigma Chemical Co.) were added to each well at concentrations previously shown to produce maximum stimulation of equine lymphocytes. Control wells contained no mitogen or culture supernatant (Medium 199 was substituted for culture supernatant). Plates were incubated at 39°C in a humidified atmosphere of 5% CO_2:95% air. After 7 h, [^{3}H]thymidine (0.5 μCi/well, 6.7 Ci/mmol, New England Nuclear, Boston, MA) was added in a volume of 50 μl complete medium. Six hours later, cells were harvested onto glass microfiber filter paper by use of a semi-automatic cell harvester (Skatron, Sterling, VA). The paper was dried in an oven for 15 min, and the discs were then placed into vials with 2 ml scintillation fluid (Optifluor, Packard, Downers Grove, IL). Radioactivity was measured in a scintillation counter (Beckman, Fullerton, CA). Mean counts per minute (cpm) of unstimulated lymphocytes (no mitogen) were subtracted from all test and control (mitogen but no supernatant) wells before calculation of results. Lymphocyte viability was determined at the end of the culture procedure by trypan blue dye exclusion in wells that did not receive [^{3}H]thymidine.

Culture supernatant from each of 3 embryos and from endometrium from 3 pregnant mares was subjected to protease digestion, charcoal stripping, and heat treatment to define the nature of immunosuppressive substance(s) present. After treatment, supernatants were filter sterilized (45-μm pore size). In further experiments, supernatants were diluted, or addition of supernatant was delayed by 24 h. Lymphocyte blastogenesis assays were performed using Con A as mitogen.

Effect of Preincubation of Cells with Supernatant

Cells (2×10^7 in 0.2 ml) were incubated with 0.2 ml supernatant for 24 h at 39°C in an atmosphere of 5% CO_2:95% air. The cells were then washed twice in PBS and resuspended in complete medium at 4×10^6 viable mononuclear cells/ml. The cells were then included in a lymphocyte blastogenesis assay.

Statistical Analysis

Effect of culture supernatants on blastogenesis was analyzed by a one-way analysis of variance (ANOVA) with least significant difference mean comparisons where appropriate (calculated from cpm). Effect on blastogenesis of different treatments of culture supernatants (% of control values) was analyzed by a repeated-measures ANOVA with least significant difference mean comparisons in which, for each animal, treatment means were compared with the mean from untreated supernatant. Lymphocyte viabilities were compared by use of Student's *t*-test. All means are quoted ± SEM.

RESULTS

Figure 1 shows the effect on peripheral blood lymphocyte blastogenesis of addition of supernatant from endometria and embryos from pregnant mares compared with supernatant from endometria of nonpregnant mares. Overall, addition of culture supernatant had a significant effect on Con A-induced blastogenesis ($p<0.001$) and on PHA-induced blastogenesis ($p<0.01$). There was no significant difference in Con A-stimulated [^{3}H]thymidine incorporation between wells containing supernatant from the endometria of nonpregnant mares and control wells containing Medium 199. Culture supernatant from embryos and endometrium of pregnant mares reduced both Con A- ($p<0.001$) and PHA-stimulated ($p<0.01$ and $p<0.05$, respectively) blastogenesis. Lymphocyte viability was not affected by

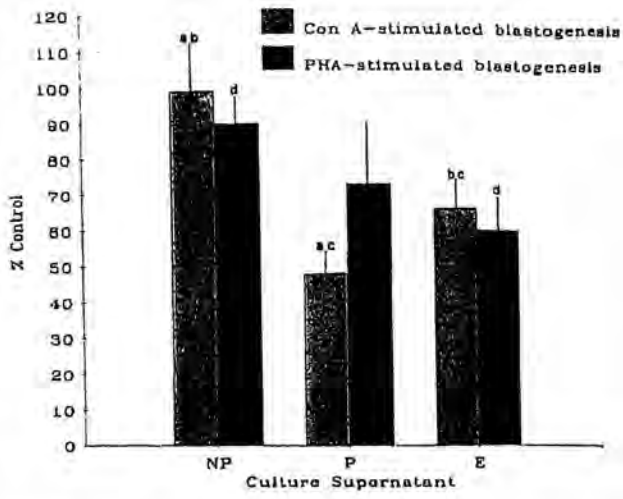

FIG. 1. Effect on lymphocyte blastogenesis of culture supernatant from embryos (*E*; n=6) and endometria of pregnant (*P*; n=6) and nonpregnant (*N*; n=6) mares 14 days after ovulation. Results are expressed as percent of cpm in mitogen-stimulated wells that did not contain supernatant. Values represent mean ± SEM determinations of quadruplicate wells. Values with similar *superscripts* are significantly different: $^{a,b}p<0.001$; $^{c}p<0.01$; $^{d}p<0.05$.

supernatant. Mean (± SEM) percentage of viability values for control and all supernatant cultures combined were 78.0 ± 6.0 and 64.7 ± 4.3, respectively.

Culture supernatant from endometrium of pregnant mares, but not from embryos, significantly ($p<0.05$) reduced blastogenesis of bovine mononuclear cells (control = 104,734 ± 5933; endometrial culture supernatant = 34,743 ± 2515; embryo supernatant = 89,038 ± 4786 cpm).

Dilution of the supernatants to 1:5 or more resulted in disappearance of the immunosuppressive activity (data not shown). Pretreatment of the supernatants with trypsin or charcoal markedly reduced suppression of lymphocyte blastogenesis (Fig. 2). Treatment of Medium 199 with trypsin and trypsin inhibitor did not significantly alter lymphocyte blastogenesis compared with untreated wells (9176 ± 1102 vs. 7730 ± 425 cpm, respectively). Heat treatment reduced the immunosuppressive activity of culture supernatants, but this reduction was significant only with the embryo culture medium (Fig. 2). When tissues had been incubated in the presence of indomethacin, immunosuppressive activity was virtually absent in 5 of the 6 culture supernatants. Addition of indomethacin itself to Medium 199 had no direct effect ($p=0.34$) on lymphocyte blastogenesis (data not shown). A 24-h delay in addition of embryo,

TABLE 1. Effect of preincubation of blood lymphocytes with culture supernatants on subsequent lymphocyte reactivity.*

Preincubation medium[a]	Nonstimulated[b] (cpm)	Con-A-induced blastogenesis (cpm)	% Viable cells after preincubation procedure
Supernatant of endometrium from 3 pregnant mares	527 ± 151	18,379 ± 499	29
	1473 ± 87	32,103 ± 1608	39
	2592 ± 380	35,755 ± 3494	67
Culture supernatant from 3 embryos	1272 ± 103	8139 ± 577	48
	495 ± 153	21,541 ± 1231	30
	1374 ± 169	34,168 ± 5464	60
Culture medium (Medium 199)	402 ± 31	9355 ± 1302	60

[a]Con A-induced blastogenesis was significantly ($p<0.05$) greater in cells preincubated with endometrial supernatant than with culture medium. None of the other treatments was significantly different from preincubation with culture medium.

[b]Nonstimulated wells received no mitogen.

*Each result is the mean of quadruplicate wells.

but not endometrial, culture supernatant significantly increased incorporation of [^{3}H]thymidine compared with wells in which supernatant was added at the start of culture (Fig. 3).

Preincubation of cells with endometrial, but not embryo, supernatants for 24 h resulted in significantly ($p<0.05$) increased incorporation of [^{3}H]thymidine in wells with mitogen (Table 1). There were no significant differences in cpm in nonstimulated wells. Incorporation of thymidine was not related to the degree of cell death that occurred during the preincubation period of cells with culture supernatant.

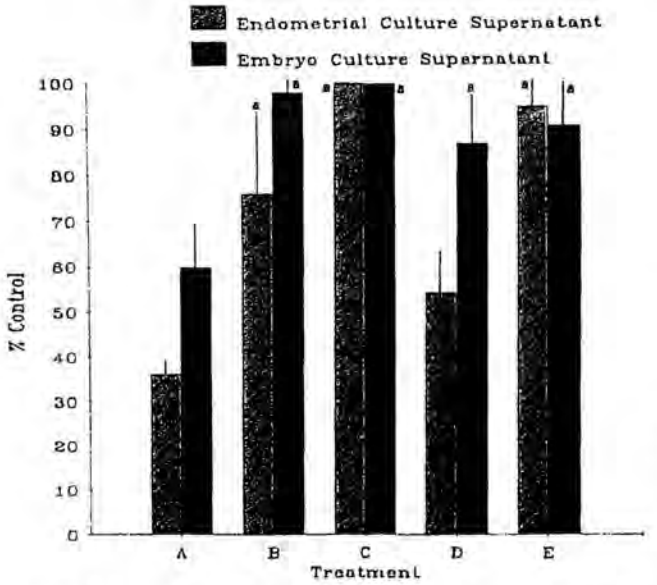

FIG. 2. Effect on Con A-stimulated blastogenesis of immunosuppressive substance(s) in culture supernatant from endometrium of 3 pregnant mares and from 3 embryos following various treatments. Results are expressed as percent of cpm from wells to which no supernatant was added. Each column represents the mean ± SEM of quadruplicate wells. *A*, No treatment. *B*, Trypsin treatment: supernatant (0.5 ml) was incubated with trypsin (10 μl, 10 mg/ml) for 4.5 h at 37°C followed by addition of trypsin inhibitor (2-fold excess). *C*, Charcoal extraction: supernatant (0.75 ml) was incubated with charcoal (25 mg) for 30 min at room temperature, then centrifuged at 2000 × *g* for 15 min. *D*, Heat treatment: supernatants were incubated at 60°C for 30 min. *E*, Indomethacin treatment: tissues were cultured in the presence of indomethacin. [a]$p<0.01$, Level of significance compared with untreated supernatant.

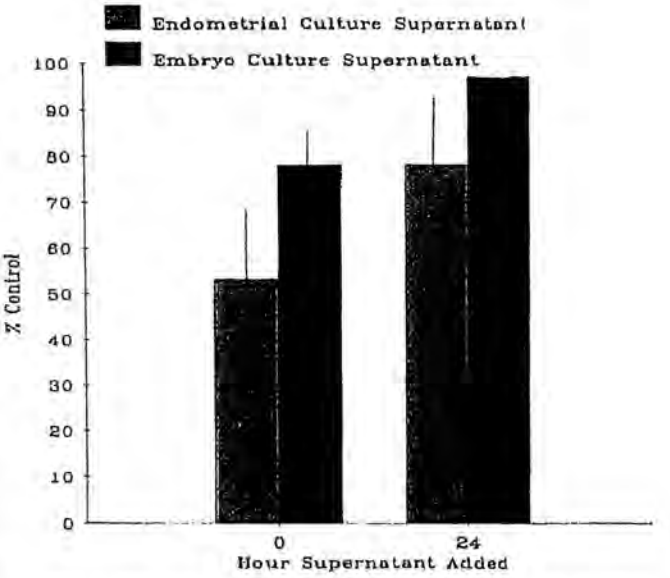

FIG. 3. Effect on Con A-stimulated blastogenesis of addition of culture supernatant (3 endometrial and 3 embryo) 24 h after the start of the blastogenesis assay. Results are expressed as a percent of cpm from wells containing no culture supernatant. Values represent mean ± SEM determination of quadruplicate culture wells.

DISCUSSION

This study has demonstrated the release of a lymphocyte inhibitory factor(s) by endometrial and embryonic tissues collected 14 days after ovulation. In the mare, as has been previously reported in other species (Fisher et al., 1985; Murray et al., 1987; Segerson, 1988), this immunosuppressive effect is not due to a cytotoxic effect of the culture supernatant on the lymphocytes.

Both Con A and PHA preferentially stimulate T cells (Schechter, 1980). It is likely that when cells were stimulated with Con A, a significant number of B cells also responded. In other species, Con A is not as specific in stimulation of T cells as is PHA (Kristensen et al., 1982). However, responses to each mitogen were not significantly different in the present study. The active substance(s) in culture supernatant from horse embryos and endometrium exhibited similar properties when subjected to various treatments. The substance(s) was significantly reduced or removed by treatment with charcoal or trypsin, and its synthesis and/or release appeared to be inhibited when the tissue was cultured in the presence of indomethacin. Heating at 60°C for 30 min significantly reduced the activity of supernatant from embryos, indicating that some heat-labile complement components or other proteins may be involved.

In cattle and sheep, trophoblastic proteins that appear to share structural and functional characteristics with alpha-interferons have been identified (Imakawa et al., 1989). Purified ovine trophoblast protein-1 can inhibit the incorporation of [^{3}H]thymidine into mitogen-stimulation ovine lymphocytes (Roberts et al., 1989). Analogous proteins have not yet been isolated from secretions of the endometrium of pregnant mares. An immunosuppressive substance produced by murine and human decidual cells with properties similar to those of the one in the present study was identified as PGE_2 (Tawfik et al., 1986; Parhar et al., 1988). Suppression by PGE_2 from human decidual cells resulted from down-regulation of interleukin-2 (IL-2) receptor development on lymphocytes and also inhibition of IL-2 production (Lala et al., 1988). PGE_2 has also been implicated as a lymphocyte inhibitory factor in ruminants (Low and Hansen, 1988), and PGE_2 suppresses equine lymphocyte blastogenesis when added at physiological concentrations (Watson, unpublished data). Although the data in the present study are highly suggestive of involvement of prostaglandins in the observed immunosuppressive activity and PGE_2 is associated with suppression of lymphocyte blastogenesis, there was no difference in concentrations of PGE_2 measured in supernatants from pregnant and nonpregnant endometrium (Watson and Sertich, 1989). Addition of indomethacin to culture supernatants after incubation did not affect blastogenesis. Therefore, it is unlikely that a proteinaceous factor was present that was suppressing blastogenesis by altering production of prostaglandins by the cultured mononuclear cells. It is possible that cyclooxygenase metabolites of an arachidonate other than PGE_2 may have been involved in immunosuppression by endometrial culture supernatant. Interestingly, when addition of embryo culture supernatant was delayed by 24 h, the suppressive effect on lymphocyte blastogenesis was eliminated. It has been reported that PGE_2 must be present within 4 h of mitogen addition for maximum suppressive effect on blastogenesis of bovine lymphocytes (Muscoplat et al., 1979). Therefore, perhaps PGE_2 was involved in the immunosuppression observed with embryo culture medium.

Depletion of proteins by trypsin digestion reduced or eliminated the immunosuppressive effect of supernatants. Similar results obtained with bovine embryo culture medium have led to the conclusion that inhibition of lymphocyte proliferation was due, at least in part, to a peptide (French and Northey, 1983; Fisher et al., 1985). It seems likely that proteins are involved in the immunosuppression observed in the present experiment.

The lymphocyte inhibitory factors in equine endometrial culture supernatant also suppressed bovine cells. Cross-species suppression by uterine factors has also been reported in rabbits (Pandian et al., 1988) and pigs (Allen et al., 1981), but not in cattle (French and Northey, 1983).

Inhibition of lymphocyte blastogenesis by supernatants may have been due in part to blockade of lymphocyte receptor sites for mitogens by proteins present in the culture supernatants. The possibility also exists that substances in the supernatant inactivated or bound to the mitogens, thus making them unavailable for attachment to the cell surface. Con A can be removed from lymphocytes by addition of alpha-methylmannosidase after 20 h of incubation without any reduction in blastogenic response (Gunther et al., 1974). In our study, late addition of embryo culture supernatant eliminated its immunosuppressive effect. It was, therefore, not possible to exclude the possibility that embryo culture supernatant was exerting its effect by interfering with Con A-lymphocyte interactions. However, this explanation would not account for elimination of suppression by culturing the tissues in the presence of indomethacin or after treatment of the supernatants with charcoal.

Progesterone has been shown to induce immunosuppressive activity in uterine secretions from nonpregnant heifers (Segerson et al., 1986) and in culture supernatant from human endometrial explants (Wang et al., 1988). Furthermore, it has been shown that allogeneic skin grafts survive in the uterine lumen of progesterone-treated ewes (Hansen et al., 1986). However, progesterone itself suppressed in vitro blastogenesis of ovine and bovine lymphocytes only at concentrations 100 times higher than those found in peripheral blood during pregnancy (Low and Hansen, 1988). When administered to mares at normal physiological doses, progesterone does not inhibit blastogenesis of lymphocytes collected from the blood of these animals (Watson, 1987). Also, the endometrium of the nonpregnant mares sampled in this study was probably still under the influence of progesterone 14 days after ovulation, but supernatant from these incubations did not affect blastogenesis. Receptor numbers and peripheral blood concentrations of progesterone are similar in early pregnant and nonpregnant mares (Watson, unpublished data). Unless progesterone had an effect on the endometrium from early pregnant mares different from that on endometrium from nonpregnant mares, it seems unlikely that progesterone itself was directly responsible for, or that it induced, the lymphocyte inhibitory activity of endometrial culture supernatant.

It is interesting that preincubation of lymphocytes with culture supernatant stimulated lymphocyte reactivity in both the presence and absence of mitogen. Since the effect of addition of supernatant on lymphocyte blastogenesis is almost certainly the end result of a complicated mixture of stimulatory and inhibitory factors, perhaps the lymphocytes were directly stimulated by the supernatant. It is possible that immunomodulatory products such as lymphokines that influence subsequent lymphocyte function during preincubation may eventually be identified in the uterus of the pregnant mare.

The results show that some heat-labile complement components or other proteins or prostaglandins released by embryos and by the endometrium of early pregnant mares suppress lymphocyte reactivity in vitro. It is suggested that these factors may be important in suppressing rejection of the conceptus allograft.

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Regulation of mitogen- and TCGF-induced lymphocyte blastogenesis by prostaglandins and supernatant from equine embryos and endometrium

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Immunosuppressive substances which interfere with lymphocyte blastogenesis are released in vitro by embryos and endometrium from mares in early pregnancy. Immunosuppression was not evident when tissues were cultured in the presence of indomethacin (a prostaglandin-synthesis inhibitor). Various prostaglandins (PGs) were added to equine lymphocytes and lymphocyte proliferation was measured after the addition of concanavalin A (Con A) or phytohaemagglutinin A (PHA). PGE_2 and $PGF_{2\alpha}$ inhibited Con A-induced blastogenesis down to final concentrations of $1{\cdot}8\times10^{-9}$ M and $1{\cdot}3\times10^{-6}$ M, respectively. Other PGs tested (6-keto-$PGF_{1\alpha}$ and 13,14-dihydro-15-keto-$PGF_{2\alpha}$) did not affect Con A-induced blastogenesis. PHA-induced blastogenesis was inhibited by concentrations down to $1{\cdot}8\times10^{-9}$ M PGE_2, $3{\cdot}3\times10^{-7}$ M $PGF_{2\alpha}$ and $2{\cdot}8\times10^{-4}$ M 6-keto-$PGF_{1\alpha}$. At all concentrations, 13,14-dihydro-15-keto-$PGF_{2\alpha}$ only slightly reduced PHA-induced blastogenesis. Therefore, PGE_2 was the only prostaglandin tested which, at physiological concentrations, significantly inhibited incorporation of [^{3}H] thymidine. The mechanism of PGE_2-mediated suppression was studied by adding PGE_2 and T cell growth factors (TCGF) to TCGF-responsive lymphocytes. PGE_2 reduced the TCGF-mediated blastogenic response in a dose-dependent manner. Furthermore, culture supernatant from embryos and endometrium from 14-day pregnant mares inhibited lymphocyte blastogenesis induced by TCGF. These results show that PGE_2 interferes with lymphocyte blastogenesis by TCGF-dependent mechanisms. It is suggested that the PGE_2 present in the uterus of the early pregnant mare may be one of the factors involved in immunosuppression at the time of maternal recognition of pregnancy.

DURING pregnancy, the dam is exposed to alloantigens present on the surface of the developing conceptus. The equine conceptus is surrounded by an acellular capsule from day 6·5 to day 21 of pregnancy (Betteridge et al 1982, Enders et al 1988). However, the presence of this capsule does not appear to mask embryonic antigens (White et al 1988). Successful pregnancy is dependent upon regulation of the maternal immune system to prevent rejection of the conceptus allograft. Recently, prostaglandin (PG) E_2 has been implicated as an important immunoregulator during early pregnancy in the mouse and human (Tawfik et al 1986, Lala et al 1988, Parhar et al 1988). PGE_2 has long been acknowledged to have immunomodulatory properties, including inhibition of lymphocyte proliferation (Muscoplat et al 1979), cytolysis (Trofatter and Daniels 1979) and lymphokine production (Gordon et al 1976, Chouaib et al 1985).

Prostaglandins are produced by the conceptus and endometrium in the pregnant cow (Lewis et al 1982, Shemesh et al 1984), the ewe (Evans et al 1982, Rawlings and Hyland 1985) and the mare (Watson and Sertich 1989). PGE_2 and PGI_2 are thought to have a luteotrophic role in early pregnancy in ruminants (Hueckstead and Weems 1978, Milvae and Hansel 1983), however, administration of exogenous PGE_2 in the mare does not extend the lifespan of the corpus luteum (Sharp et al 1989a). Accordingly, the role of prostaglandins during early pregnancy in the mare is not clear.

Conditioned medium from embryos and endometria of pregnant mares suppressed mitogen-stimulated lymphocyte blastogenesis in vitro (Watson 1990). However, conditioned medium from incubations performed in the presence of indomethacin, a prostaglandin-synthesis inhibitor, was not immunosuppressive. These findings suggest the involvement of prostaglandins as inhibitory factors.

The present study investigated the mechanism of inhibition of lymphocyte blastogenesis by culture supernatant from embryos and endometrium from pregnant mares by adding a source of T cell growth factors (TCGF) to TCGF-responsive lymphocytes

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suspended in culture supernatant and measuring subsequent incorporation of [^{3}H] thymidine. The effect of prostaglandins on blastogenesis of equine lymphocytes was also studied.

Materials and methods

Mononuclear cell isolation

Blood from healthy horses was collected into evacuated tubes containing EDTA and centrifuged at 2000 *g* for 10 minutes. The buffy coat was removed and washed once in phosphate buffered saline (PBS). The cells were resuspended in PBS, layered on to Histopaque 1077 (Sigma Chemical) and centrifuged at 400 *g* for 30 minutes. Cells at the interface (> 95 per cent mononuclear cells) were then removed and washed twice in PBS before being resuspended in complete medium (RPMI-1640 containing 10 per cent fetal calf serum, 100 units penicillin plus 100 μg streptomycin ml^{-1}, 2 mM L-glutamine and 10^{-5} M 2-mercaptoethanol) at a concentration of 4×10^6 cells ml^{-1}. Viability was assessed by exclusion of trypan blue dye. Using this method, more than 95 per cent of isolated cells were viable.

Lymphocyte blastogenesis assay

This was performed as described previously (Watson 1990). Concanavalin A (Con A, 50 μl, 20 μg ml^{-1}) or phytohaemagglutinin A (PHA, 50 μl, 12·5 μg ml^{-1}) and prostaglandins (50 μl) were added to cells (50 μl, 4×10^6 ml^{-1}). Control wells did not contain mitogen or prostaglandins. After a 72 hour incubation at 39°C in a humidified atmosphere of 5 per cent carbon dioxide:95 per cent air, [^{3}H] thymidine (0·5 μCi per well, specific activity 6·7 Ci mmol^{-1}, New England Nuclear) was added in 50 μl of complete medium. Six hours later, the cells were harvested on to glass fibre discs using a semi-automated cell harvester (Skatron). Mean counts per minute (cpm) of unstimulated wells were subtracted from all other wells before calculation of results.

TCGF-induced blastogenesis

TCGF was generated and assayed by the procedure described by Oldham and Williams (1984). Briefly, TCGF produced by Con A-stimulated mononuclear cells was harvested and added at a 1:2 dilution to wells on a microtitre plate. It is likely that most of the TCGF activity could be attributed to interleukin-2 (Oldham and Williams 1984). TCGF-responsive cells were produced by culturing peripheral blood mononuclear cells with Con A (5 μg ml^{-1}) at 39°C in an humidified atmosphere of 5 per cent carbon dioxide:95 per cent air for four days. The Con A blasts were then washed three times in PBS and resuspended in complete medium. Con A blasts (50 μl; 4×10^5 ml^{-1}) were added to wells containing PGE_2 (100 μl); PGE_2 was not added to control wells. Serial twofold dilutions of TCGF (100 μl) were added to wells to a final dilution of 1:64. After 24 hours, 1 μCi [^{3}H] thymidine was added to each well and the cells were harvested 18 hours later. To ensure that no Con A remained in the cell supernatant, wells containing freshly isolated peripheral blood mononuclear cells were included in each assay. Wells that contained no TCGF were also included and cpm in these wells were subtracted before calculation of results as a percentage of cpm in wells lacking PGE_2.

Embryo and endometrial culture supernatant

Embryos and endometrium were collected from pregnant mares 14 days after ovulation and cultured for 20 hours as previously described (Watson and Sertich 1989). The supernatants were stored at −70°C and filter sterilised (45 μm) before being added (50 μl) to wells containing Con A blasts and TCGF. The degree of [^{3}H] thymidine incorporation was expressed as a percentage of cpm in control wells which did not contain supernatant.

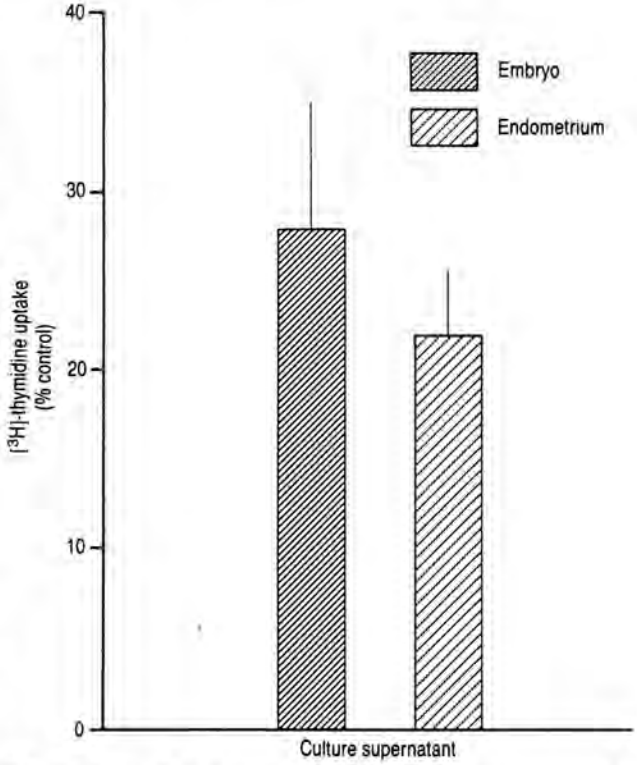

FIG 1: Effect of culture supernatant from embryos or endometrium collected from pregnant mares 14 days after ovulation on TCGF-induced blastogenesis. Results are expressed as a percentage of control wells which did not contain supernatant. Control = 11,426 ± 1134 cpm

Statistical analyses

Data (cpm) were analysed by a one-way analysis of variance (culture supernatants) or by a repeated measures analysis of variance (prostaglandin treatments) with least significant difference mean comparisons ($P < 0{\cdot}01$) where appropriate.

Results

Addition of embryo and endometrial culture supernatant to TCGF-responsive lymphocytes significantly ($P < 0{\cdot}001$) reduced incorporation of [^{3}H] thymidine after stimulation with TCGF (Fig 1). Neither 6-keto-$PGF_{1\alpha}$ nor 13,14 dihydro-15-keto-$PGF_{2\alpha}$ (PGFM) significantly influenced lymphocyte blastogenesis stimulated by Con A (Fig 2). Con A-induced blastogenesis was significantly reduced by higher doses of $PGF_{2\alpha}$ ($\geq 1{\cdot}3 \times 10^{-6}$ M). PGE_2 significantly reduced blastogenesis ($P < 0{\cdot}001$) at all concentrations of Con A and PHA down to and including $1{\cdot}8 \times 10^{-9}$ M (data not shown). Low concentrations of PGFM and high concentrations of 6-keto-$PGF_{1\alpha}$ significantly suppressed PHA-induced blastogenesis (Fig 3). $PGF_{2\alpha}$ significantly suppressed PHA-induced blastogenesis at concentrations of $3{\cdot}3 \times 10^{-7}$ M and higher.

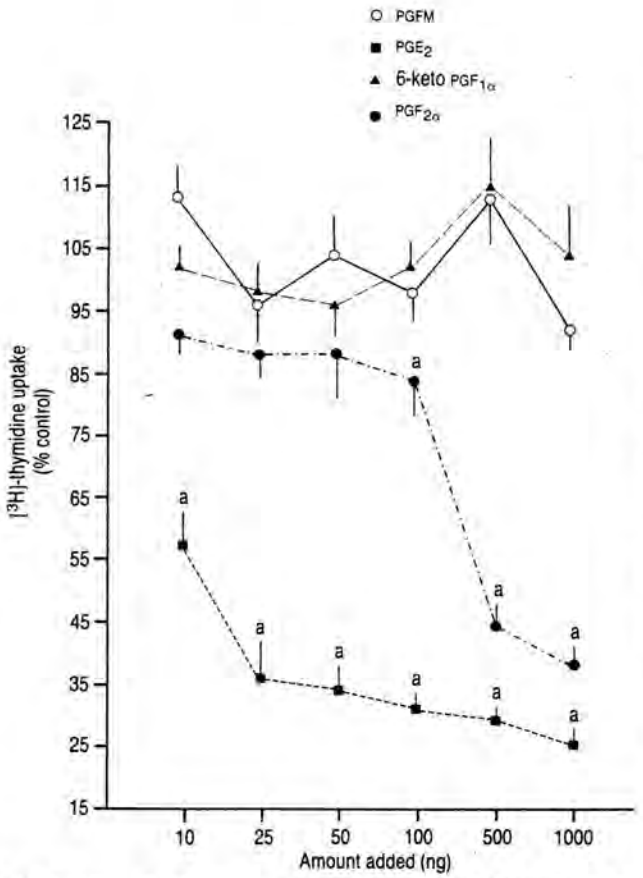

FIG 2: Effect of prostaglandins on Con A-induced blastogenesis of equine lymphocytes. Data are presented as a percentage of [^{3}H] thymidine incorporation in wells without prostaglandins. Values with superscripts are significantly different ($P < 0{\cdot}01$) from the control. Each result represents the mean (± SEM) of six wells. (10 ng $PGE_2 = 1{\cdot}8 \times 10^{-7}$ M; 10 ng $PGF_{2\alpha} = 1{\cdot}3 \times 10^{-7}$ M; 10 ng 6-keto-$PGF_{1\alpha} = 5{\cdot}5 \times 10^{-6}$ M; 10 ng PGFM = $5{\cdot}3 \times 10^{-6}$ M). Control = 28,839 ± 2226 cpm

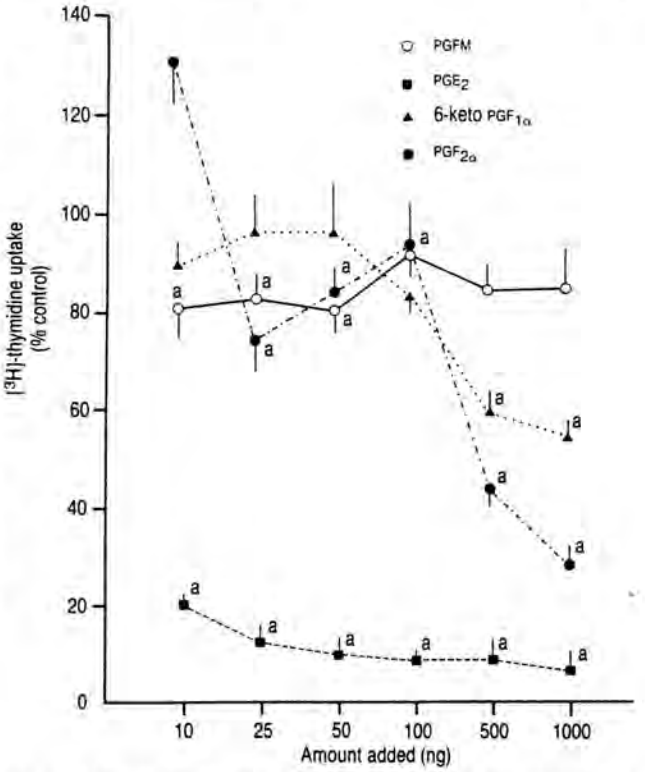

FIG 3: Effect of prostaglandins on PHA-induced blastogenesis of equine lymphocytes. Data are presented as a percentage of [^{3}H] thymidine incorporation in wells without prostaglandins. Values with superscripts are significantly different ($P < 0{\cdot}01$) from the control. Each result represents the mean (± SEM) of six wells. Control = 10,099 ± 764 cpm

When PGE_2 was added to TCGF-responsive lymphocytes in the presence of a source of TCGF (Fig 4), blastogenesis was significantly reduced at all concentrations tested ($P < 0{\cdot}001$). None of the treatments had any effect on lymphocyte viability (data not shown).

Discussion

The authors have shown that, of the prostaglandins tested, PGE_2 was the most effective inhibitor of blastogenesis of equine lymphocytes. PGE_2 inhibited blastogenesis at concentrations down to 10^{-9} M. PGE_2 has also been reported to inhibit PHA-induced blastogenesis at similar concentrations in cattle, sheep (Low and Hansen 1988), humans (Goodwin et al 1977) and mice (Goodwin and Cueppens 1983). Concentrations of PGE_2 and $PGF_{2\alpha}$ in the endometrium of mares in early gestation are approx-

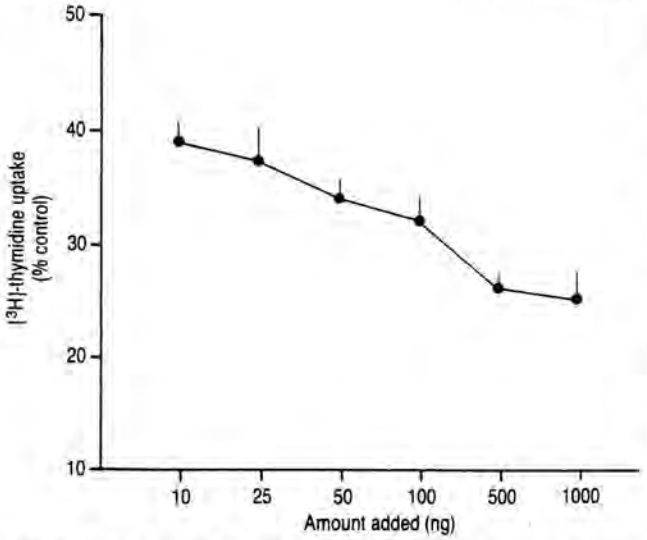

FIG 4: Effect of PGE_2 on TCGF-induced lymphocyte blastogenesis. Data are presented as a percentage of [^{3}H] thymidine incorporation in wells without PGE_2. All values were significantly lower ($P<0.001$) than the control. Each result represents the mean (± SEM) of six wells. Control = 8189 ± 686 cpm

imately 5×10^{-7} M (Watson and Sertich 1989). Therefore, PGE_2 was a highly effective inhibitor at physiological concentrations, whereas $PGF_{2\alpha}$ was an effective (>50 per cent) inhibitor only at pharmacological concentrations. No significant differences in concentrations of PGE_2 were detected in culture supernatant from 24-hour incubations of endometrium from pregnant and non-pregnant mares at day 14 after ovulation (Watson and Sertich 1989). However, the finding that incubation of endometrium in the presence of indomethacin reversed the immunosuppressive effects of culture supernatant (Watson 1990) was highly suggestive of a role for prostaglandins in the inhibition of lymphocyte proliferation. It seems likely, therefore, that another as yet unidentified substance that is absent from the non-pregnant endometrium is acting synergistically with prostaglandins. It appears that, unlike the cow, sow and ewe endometrium, the mare endometrium does not produce an interferon-like protein at this stage of pregnancy (Sharp et al 1989b). In other species, this substance has been shown to inhibit lymphocyte blastogenesis (Murray et al 1987, Newton et al 1989). Slightly different results were obtained with each of the mitogens. In other species, Con A is not as specific in stimulation of T cells as is PHA (Kristensen et al 1982). B cells may, therefore, have contributed to the slight difference in response to each mitogen.

PGE_2 is reported to have a suppressive effect on interleukin-2-induced proliferation of lymphocytes from cattle and sheep (Low and Hansen 1988). In the present study, both PGE_2 and culture supernatant from embryos and endometrium from pregnant mares inhibited TCGF-induced lymphocyte proliferation. The degree of inhibition shown by culture supernatant that contained less PGE_2 per well (0·1 ng; Watson and Sertich 1989) than the lowest dose tested was greater than could be attributed entirely to PGE_2. Previous work has shown that it is unlikely that prostaglandins are solely responsible for the immunosuppressive qualities of culture supernatant from embryos and endometrium of pregnant mares (Watson 1990), but rather that other substances, possibly proteinaceous, also contribute to immunosupppression. Progesterone at pharmacological rather than physiological concentrations is immunosuppressive to human (Clemens et al 1979) and sheep (Staples et al 1983) but not cattle (Murray and Chenault 1982) lymphocytes. Dioestrous concentrations of progesterone did not inhibit blastogenesis of blood lymphocytes from mares (Watson 1987). Although it is likely that local uterine concentrations of progesterone are considerably higher than circulating concentrations during dioestrus and early pregnancy, previous results using culture supernatant from the endometrium of pregnant (suppressive to lymphocyte blastogenesis) and non-pregnant dioestrous (no effect on lymphocyte blastogenesis) mares suggest that intrauterine progesterone was not implicated in suppression of blastogenesis (Watson 1990).

Addition of PGE_2 to human lymphocytes did not interfere with lymphokine-induced blastogenesis of preactivated lymphocytes already expressing interleukin-2 receptors, but interleukin-2 receptor expression and interleukin-2 production were impaired (Vercammen and Weppens 1987). Furthermore, it has been shown that PGE_2 secreted by first trimester human decidual cells also inhibited interleukin-2 receptor generation and production by lymphocytes in vitro (Lala et al 1988, Parhar et al 1989). In the present study, suppression of lymphokine-induced blastogenesis occurred in the presence of PGE_2. Thus PGE_2 may interfere with early events in activation of equine T cells during the lymphokine-dependent period of lymphocyte proliferation by reducing the responsiveness of lymphocytes to lymphokines.

In conclusion, the present study suggests that PGE_2 may be one of the substances which contributes to the inhibition of proliferation of maternal lymphocytes within the endometrium. More work is needed to confirm the action of PGE_2 in vivo during early pregnancy in the mare.

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EFFECT OF PROSTAGLANDIN F2α ON RELEASE OF PROGESTERONE AND LEUKOTRIENE B-4 BY CELLS FROM CORPORA LUTEA OF MARES

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ABSTRACT

Corpora lutea were recovered from mares either 4 to 5 days or 12 to 13 days after ovulation. Mixed populations of luteal cells were prepared by collagenase digestion and were incubated for 24 h in the presence or absence of prostaglandin (PG) F-2α (250 ng/ml). PGF-2α significantly (P = 0.03) reduced progesterone secretion by cells from late diestrous corpora lutea and tended (P = 0.06) to reduce secretion by early diestrous cells. PGF-2α had no significant effect on leukotriene B-4 (LTB-4) production by cells from early diestrous corpora lutea, but significantly (P = 0.03) increased LTB-4 production by late diestrous luteal cells. It seems possible that LTB-4 could play a role as an intermediary in the action of PGF-2α in luteolysis in the mare.

INTRODUCTION

There is strong circumstantial evidence that prostaglandin (PG) F-2α is the luteolysin in the mare (1,2,3). The mode of action of the luteolytic effect of PGF-2α is not well understood but it is known that there are specific high-affinity receptors for PGF-2α in membrane fractions prepared from equine corpora lutea (4,5) and that receptor numbers increase in late diestrus. There is no information, however, on the effect of PGF-2α on progesterone synthesis by isolated equine luteal cells.

Recent evidence shows that the lipoxygenase products of metabolism of arachidonic acid may act as intermediaries in the luteolytic action of PGF-2α. Addition of lipoxygenase products to bovine luteal cells *in vitro* resulted in a reduction in progesterone synthesis and administration of an inhibitor of lipoxygenase pathways to cows delayed luteolysis (6). Furthermore, luteal cells from cattle and sheep synthesize lipoxygenase products *in vitro*, and this synthesis is increased in ewes by systemic administration of PGF 2α prior to removal of the corpora lutea (6,7).

The present study investigated whether lipoxygenase products might be involved in luteolysis in the mare by measuring the effect of PGF-2α on production of progesterone and leukotriene B-4 (LTB-4) by equine luteal cells *in vitro*.

MATERIALS AND METHODS

Seven Standardbred and Thoroughbred horse mares aged 3 to 12 years were used. Mares were teased daily with a stallion to detect onset of estrus and then the mares' ovaries were examined daily by ultrasonography per rectum to determine the day of ovulation (designated Day 0). In 4 of the mares (n=4 corpora lutea), the ovary containing the corpus luteum was removed at Day 4 or 5 and in 5 of the mares (n=7 corpora lutea), the ovary containing the corpus luteum was removed at Day 12 or 13. In 2 of the mares, one ovary was removed during each of two successive cycles.

Ovariectomy

The procedure has been described previously (8). Mares were sedated with xylazine and analgesia was obtained after intravenous administration of butorphanol. After thorough cleansing of the perineal region, the ovary was removed using an ecraseur via an incision in the cranial vagina. The ovarian ligament was anesthetized by local application of lidocaine before being crushed by the ecraseur.

Preparation of luteal cells

The ovaries were suspended in Medium 199 on ice until they were transported to the laboratory. After removal of the corpus luteum from the ovary, the capsule was removed. Cells were dissociated by collagenase digestion as previously described (8) and the cells were suspended in medium (Medium 199 containing 50 units penicillin, 50 ug streptomycin, 10% newborn calf serum, and 4.7 ul 7.5% bicarbonate per ml) at a concentration of 5 x 10^5 cells/ml. Corpora lutea obtained from mares in early diestrus contained 46 $\pm$ 4.7% large cells (mean $\pm$ SEM) whereas corpora lutea from mares in late diestrus contained 24.3 $\pm$ 2.0% (8).

Incubations

Luteal cells (4 ml) were incubated in 60 mm sterile tissue culture dishes (Becton Dickinson, Lincoln Park, NJ) for 24 h at 37°C in an humidified atmosphere of 5% CO-2 : 95% air. Cultures were performed in triplicate and each experiment contained control and PGF-2α-treated plates. Prostaglandin F-2α was added to give a final concentration of 250 ng/ml. After incubation, the supernatants were centrifuged at 2,000 *g* for 10 min to pellet any non-adherent luteal cells and were stored in aliquots at -20°C until assay.

Radioimmunoassays

Concentration of progesterone in unextracted culture supernatant was measured as previously described (8).

Leukotriene B-4 was measured in unextracted supernatant using a commercially available kit (New England Nuclear, Boston, MA). A tritiated tracer was employed. Cross reactivities of the antiserum were with 5,12-diHete 3.6%, 20-OH-LTB-4 1.3%, 6-transLTB-4 1.0%, LTD-4 11.6% and LTE-4 3.3%. Inhibition curves obtained with standards and serial dilution of culture supernatant were parallel. Recovery of known quantities of LTB-4 from spiked culture supernatant was 98 $\pm$ 4.8%. Sensitivity of the assay was 12.5 pg/tube. The intra-assay coefficient of variation was 8%. All samples were included in one assay.

Statistical analysis

Differences in hormone concentrations between control and treated cultures were analyzed using a one-tailed Wilcoxon Signed Rank Test. Differences in hormone concentrations between corpora lutea collected at Day 4-5 or Day 12-13 were assessed by a Student's *t* test.

RESULTS

Luteal cells isolated from corpora lutea obtained during early or late diestrus did not produce significantly different concentrations of progesterone or LTB-4 (Fig. 1). Addition of PGF-2∝ to incubation medium significantly reduced secretion of progesterone from cells from late diestrous corpora lutea ($P = 0.03$) and tended to reduce secretion from cells from early diestrous corpora lutea ($P = 0.06$). Secretion of LTB-4 by cells from early diestrous corpora lutea was not affected by PGF-2∝ ($P = 0.31$) whereas there was a small but significant increase in LTB-4 secreted by cells from late diestrous corpora lutea ($P = 0.03$).

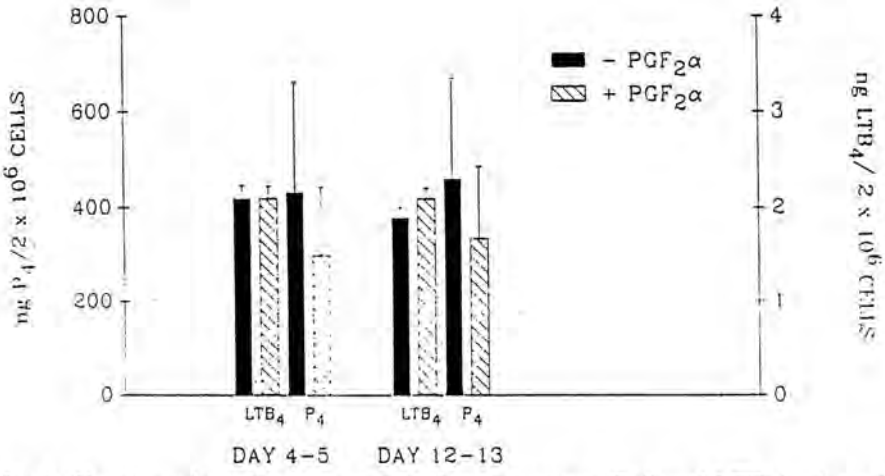

Fig.1. Concentrations (mean ± s.e.m.) of progesterone (P-4) and LTB-4 released by cells from Day 4-5 or Day 12-13 corpora lutea after incubation with or without 250 ng PGF-2∝/ml.

DISCUSSION

This study has shown that, although *in vitro* secretion of progesterone and LTB-4 by luteal cells does not vary between early and late diestrus, PGF-2∝ decreases secretion of progesterone by luteal cells and stimulates secretion of LTB-4 by late but not early diestrous luteal cells.

The equine corpus luteum consists of at least two morphologically distinct steroidogenic cell populations which differ in size (8,9,10). The proportion of large and small cells varies through the cycle with small cells predominating in late diestrus (8,9). In other species, differences exist between the actions of gonadotrophins and prostaglandins on separated large and small luteal cells. PGF-2∝ added to separated ovine luteal cells had no effect on progesterone synthesis by small cells, but inhibited production by large cells (11,12,13). By contrast, PGF-2∝ stimulated progesterone synthesis in small bovine luteal cells and did not affect progesterone synthesis in large cells (14). The overall hormone secretion by mixed luteal cell populations depends on the proportion of large and small cells present.

PGF-2α has been shown to have no effect on progesterone secretion by mixed populations of bovine luteal cells probably due to the relatively greater rate of secretion of progesterone by large luteal cells (14,15). In the present study, PGF-2α had a greater negative effect on progesterone production by cells collected from late diestrus corpora lutea than on cells from early diestrus corpora lutea. This is probably due to changing populations of cells between these two stages of diestrus.

The importance of LTB-4 production by equine luteal cells is not known. In the ewe, LTB-4 production by luteal cells was found to increase after administration of PGF-2α to ewes. It was not possible to demonstrate this effect *in vitro*, however (7). Eosinophils migrate into ovine luteal tissue after administration of luteolytic doses of PGF-2α (16) and it has been hypothesized that LTB-4 exerts an effect on luteolysis by attracting eosinophils to the corpus luteum which then secrete cytotoxic substances (7). The detection of a significant, albeit small, increase in concentrations of LTB-4 after addition of PGF-2α to luteal cells from late diestrous corpora lutea suggests a role for LTB-4 in luteolysis of the equine corpus luteum. Because of the mixed cell populations used in this study, it is not possible to determine the source of the LTB-4 within the corpus luteum. However, future studies using sorted cell populations will resolve this question.

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Short Communication

Immunolocalisation of oxytocin in the equine ovary

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Keywords: horse; oxytocin; ovary; mare

Introduction

Oxytocin has been identified in the ovaries of many species including ruminants (Wathes and Swann 1982; Rodgers *et al.* 1983), pigs (Jarry *et al.* 1990), sub-human primates (Khan-Dawood 1998), rats (Ho and Lee 1992) and women (Schaeffer *et al.* 1984). In these species ovarian oxytocin has several roles, including control of luteal regression (Flint and Sheldrick 1983) and possibly luteinisation (Aladin Chandrasekher and Fortune 1990; Eispanier *et al.* 1997), and involvement in ovulation (Viggiano *et al.* 1989).

In the mare, circulating oxytocin is reported to be high in oestrus and early dioestrus (Burns *et al.* 1991), or high in late dioestrus (Tetzke *et al.* 1987). Another paper reported that concentrations remained very low throughout the oestrous cycle (Stevenson *et al.* 1991). Short term incubation of cells from equine preovulatory follicles has failed to yield measurable concentrations of oxytocin, and follicular fluid and homogenates of corpus luteum (CL) and follicles contained only low concentrations of the peptide (Stevenson *et al.* 1991; Stock *et al.* 1995). It has been concluded, therefore, that the ovaries are not a significant source of circulating oxytocin during the oestrous cycle in mares (Stevenson *et al.* 1991). However, it is possible that even low concentrations of oxytocin within the mare ovary may have a paracrine or autocrine role in controlling ovarian function. In the present study, immunohistochemistry was used to detect and localise oxytocin within the equine ovary during the oestrous cycle and early pregnancy. The carrier protein for oxytocin, neurophysin, was also studied as its presence is taken to represent secretion of oxytocin outwith the supraopticohypophyseal system (Ciarochi *et al.* 1985).

Materials and methods

Hemiovariectomies were performed on 15 pony mares in oestrus (n = 3), early dioestrus (n = 2; Day 2), mid-dioestrus (n = 2; Day 8–10), late dioestrus (n = 3; Day 14–16), post luteolytic (n = 2; Day 16–18) and in early pregnancy (n = 3; Day 20–30). The technique used was as described previously (Watson and Sertich 1990). Functional status of the CL was confirmed by progesterone assay. A section of the wall of the preovulatory follicle or a piece of corpus luteum was placed in OCT and snap frozen in a dry ice/isopentane slurry. The tissue was then stored at -70°C.

Sections of the tissue (7 µm) were immunostained using a technique described previously (Watson and Thomson 1996). For oxytocin the primary antibody[1] was used at a dilution of 1:1,250. This antibody was specific for oxytocin and did not cross-react with other pituitary hormones or related peptides. For neurophysin, the primary antibody[2] was used at a dilution of 1:1,000.

With each batch of slides, positive (horse pituitary) and negative controls (substitution of the primary antiserum by nonimmune serum) were included. Preabsorption of the anti-oxytocin antibody with oxytocin (500 µg/ml) eliminated the immunostaining, confirming the specificity of the immunohistochemical reaction.

Results

Immunostaining for oxytocin was absent from theca and granulosa cells in preovulatory follicles. There was also no positive staining in the organising Day 2 corpus luteum. By mid-dioestrus positive staining was present in the small cells lying in the trabeculae of the corpus luteum and in between the large cells (Fig 1). No staining was seen in the large luteal cells themselves. The pattern of staining remained the same in late dioestrus but there appeared to be fewer positively staining cells. After luteolysis, staining was present in only a few cells in the CL. No staining was present in the CL obtained from pregnant mares. The ovarian stroma did not appear to contain oxytocin, but blood vessel walls stained positively. Neurophysin was not detected in any of the ovarian structures or in the ovarian stroma itself.

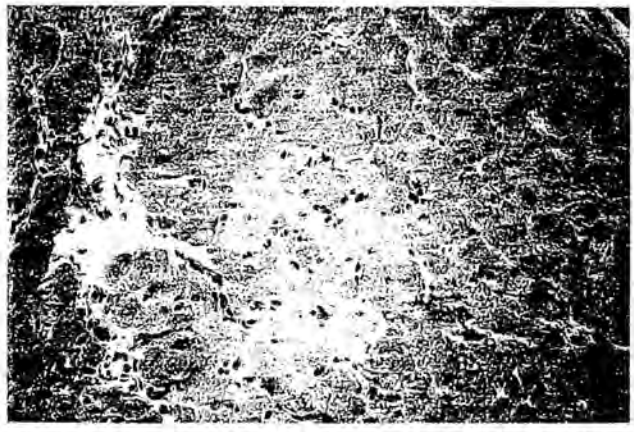

Fig 1: Day 8 corpus luteum incubated with anti-oxytocin primary antibody. Positive staining is present in small luteal cells scattered between the large luteal cells, which are negative x 20.

Negative control sections consistently failed to demonstrate any positive staining. Positive staining was detected in the neurohypophysis.

Discussion

We have shown the presence of small amounts of immunoreactive oxytocin in the corpus luteum of the nonpregnant mare. The immunostaining for oxytocin was confined to the small luteal cell population which is in direct contrast to the pattern seen in ruminants and women where the majority of the large granulosa-derived luteal cells are positive (Watkins 1983; Guldenaar *et al.* 1984; Wathes *et al.* 1986; Khan-Dawood 1987; Fields *et al.* 1992). In these species the small luteal cells are negative following immunostaining for oxytocin.

The very low level of staining for oxytocin in the regressing CL is similar to that reported in the cow (Kruip *et al.* 1985; Fields *et al.* 1992) and ewe (Wathes *et al.* 1986) and the absence of staining in the CL from early pregnant mares has also been recorded in CL from pregnant cattle (Guldenaar *et al.* 1984; Fields *et al.* 1992). The absence of immunoreactive neurophysin from the mare ovary suggests that it is unlikely that oxytocin was being synthesised by the small luteal cells during the luteal phase. The presence of oxytocin within the mid-cycle CL may however indicate binding of oxytocin by receptors. Oxytocin receptors have been identified in ruminant luteal cells (Sernia *et al.* 1989; Okuda *et al.* 1992) and it is via these receptors that oxytocin is thought to influence luteal progesterone secretion in the cow. Administration of oxytocin to the mare in mid-luteal, but not early luteal phase, shortens the oestrous cycle (Goff *et al.* 1987). Oxytocin is known to act via endometrial oxytocin receptors to cause release of PGF-2α. We have previously shown that equine luteal cells also can produce PGF-2α (Watson and Sertich 1990). However, the control of its release by luteal cells is not known. The absence of immunostaining in the CL from pregnant mares might suggest that oxytocin binding is down-regulated during pregnancy.

The lack of immunostaining for oxytocin in the preovulatory follicle and the early CL confirms the results of Stock *et al.* (1995) and strongly suggests that oxytocin is not an intrafollicular hormone in the mare.

The positive immunostaining for oxytocin seen in the blood vessels is consistent with other reproductive tissues in the horse (E.D. Watson, papers in preparation) and probably represents circulating oxytocin.

In conclusion, we have identified the presence of oxytocin in the small cells of the CL of the cyclic mare. The absence of neurophysin indicates that oxytocin is not being secreted by the mare CL. However, the presence of oxytocin suggests that oxytocin binding sites may be present and that oxytocin may be involved in control of luteal function in the mare.

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[2]Vector Laboratories, Peterborough, Cambs, UK.

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Immune cell populations in the equine corpus luteum throughout the oestrous cycle and early pregnancy: an immunohistochemical and flow cytometric study

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Recent evidence indicates that the cells of the immune system and their large network of secretory products, or cytokines, play an active role in the ovary throughout the oestrous cycle. In the present study, immune cell populations (T and B lymphocytes, macrophages, granulocytes and eosinophils) and expression of major histocompatibility complex (MHC) class II were investigated in corpora lutea from mares in early (days 2–4), mid- (days 7–10) and late (days 12–14) dioestrus, the post-luteolytic phase (days 16–17) and early pregnancy. The number of T lymphocytes within the corpus luteum increased in the late luteal phase. CD4+ cells did not increase until day 16, whereas the number of CD8+ cells increased before functional luteolysis; an apparently selective luteal infiltration of CD8+ cells was observed. MHC class II expression by non-steroidogenic cells was increased in samples from days 16–17, as was the number of infiltrating macrophages. Flow cytometry revealed very low expression of MHC class II by large luteal cells at all stages of the oestrous cycle. In early pregnancy, the number of CD4+ and CD8+ cells and macrophages decreased, as did MHC class II expression, compared with mid-dioestrous samples. B cells were present in very small numbers in all samples examined. Eosinophils were similarly sparsely distributed and numbers decreased further in pregnancy. After exogenous $PGF_{2\alpha}$ administration, populations of CD4+ cells and non-specific esterase staining cells were significantly smaller than after natural luteolysis, whereas eosinophil numbers were increased compared with samples from days 16–17. However, the number of CD8+ and CD5+ cells and MHC class II expression were not significantly different from those observed after natural luteolysis. These findings indicate that populations of immune cells in the equine corpus luteum vary during its lifespan. The selective increase in CD8+ cells before functional luteolysis indicates that they have a physiological role in the regression of the corpus luteum.

Introduction

A number of studies in different animal species indicate that immune cells play an important role in cyclic ovarian activity (Bukovsky and Presl, 1979; Murdoch *et al.*, 1988; Brannstrom and Norman, 1993). The observed similarities between inflammation and ovulation, such as vascular changes and influx of various leucocytes including macrophages, prompted initial investigation of the possibility that the immune system had a role in controlling ovarian function (Bukovsky and Presl, 1979; Espey, 1980).

The corpus luteum is a heterogeneous structure, consisting of a number of different cell populations. The morphology of the equine corpus luteum is unlike that of ruminants, in that there is marked trabeculation of the tissue. This results from the collapse of the large equine pre-ovulatory follicle (mean diameter 45–50 mm) at ovulation. The trabeculae consist of extracellular matrix, fibroblasts and small cells which are thought to be of thecal origin (Harrison, 1946), and contain much of the vasculature of the corpus luteum. The steroidogenic large luteal cells of the equine corpus luteum are thought to arise solely from the granulosa layer of the follicle (Van Niekerk *et al.*, 1975).

The functional corpus luteum is a very vascular structure which means that there are leucocytes circulating through the luteal tissue throughout its lifespan. There is also a resident leucocyte population within the ovarian interstitial tissue. In a number of species, different inflammatory cell populations such as mast cells (Jones and Hsueh, 1981; Mori, 1990), macrophages (Paavola, 1979; Brannstrom and Norman, 1993), lymphocytes (Brannstrom and Norman,

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1993) and granulocytes (Pepperell *et al.*, 1992; Brannstrom *et al.*, 1993) have been observed in the ovary throughout the oestrous cycle (Bagavandoss *et al.* 1991; Standaert *et al.*, 1991; Brannstrom *et al.*, 1994a). The primary function of macrophages observed within the corpus luteum was presumed to be removal of debris by phagocytosis during regression (Paavola, 1979). Similarly, the other leucocyte populations were initially thought to be involved in removing cells during structural regression. However, recent research has focused on the possibility that these cells play a more active role in the function of the corpus luteum (Bukovsky and Presl, 1979; Murdoch *et al.*, 1988; Bagavandoss *et al.*, 1991). White blood cells, especially macrophages and granulocytes, are a rich source of an array of cytokines with pleiotropic functions, a number of which have been implicated in altering cell function in the ovary (Adashi *et al.*, 1990; Adashi, 1990; Pate and Townson, 1994). If cytokines play a physiological role in the ovary, leucocytes must be present in sufficient numbers to produce concentrations of cytokines that could influence the function of the corpus luteum. In addition, changes in immune cell populations throughout the oestrous cycle would be expected, especially at key events such as luteolysis and ovulation. Studies in women (Petrovska *et al.*, 1992; Brannstrom *et al.*, 1994a), pigs (Standaert *et al.*, 1991), rats (Brannstrom *et al.*, 1993, 1994b) and rabbits (Bagavandoss *et al.*, 1990, 1991) found variations in populations of immune cells in corpora lutea during the oestrous cycle. The number of macrophages has been reported to increase before the onset of luteolysis in some species (Bagavandoss *et al.*, 1988; Lei *et al.*, 1991), and a study in cows showed the number of immune cells that infiltrate the corpus luteum is much smaller in early pregnancy than in the non-fertile cycle (Lobel and Levy, 1968).

If immune cells and their secreted products are involved in luteal regression, there must be a trigger that activates the immune cells. Alteration in, or aberrant expression of, major histocompatibility complex (MHC) glycoproteins on luteal cell surfaces has been proposed as such a trigger. The expression of MHC class II is central to regulation of the immune response, and is limited primarily to lymphocytes and antigen-presenting cells such as macrophages and B cells. MHC class II can also be expressed by cells of non-lymphoid origin, and this aberrant expression usually indicates that the tissue is involved in an inflammatory or immune response and is thought to be an underlying cause in certain autoimmune endocrine disorders (Kuby, 1994; Roitt, 1996). Manifestation of class II molecules by non-lymphoid cells may enable these cells to become antigen-presenting, allowing them to present previously unrecognisable autoantigens to lymphocytes for the initiation or enhancement of immune responses against the target tissue. MHC class II antigens have been detected on human luteal cells (Khoury and Marshall, 1990), and increased expression has been observed on ruminant luteal cells towards the end of the luteal phase and also after $PGF_{2\alpha}$-induced luteolysis (Fairchild Benyo *et al.*, 1991; Kenny *et al.*, 1991).

$PGF_{2\alpha}$ produced by the endometrium and transported to the ovary by a counter-current mechanism of blood vessels is accepted to be the hormone responsible for the initiation of luteolysis and regression of the corpus luteum in ruminants (Inskeep and Murdoch, 1980; Niswender *et al.*, 1985; Knickerbocker *et al.*, 1988). The close apposition of ovarian and uterine blood vessels in ruminants allows transport of prostaglandin by diffusion from the uterine vein to the ovarian artery, by-passing the systemic circulation. In mares, the role of $PGF_{2\alpha}$ in luteolysis is not as clearly defined. There is no close apposition of ovarian and uterine blood vessels in mares, and any prostaglandin released by the uterus must enter the systemic circulation to reach the ovary. However, in other species most (approximately 90%) of the prostaglandin is cleared from the circulation in one passage through the lungs. Therefore, it must be assumed that lung clearance is less effective in equines, or that only extremely small quantities of $PGF_{2\alpha}$ are required at the ovary. Mares are known to be many times (approximately 10–18 times) more sensitive than cows, pigs or ewes to the luteolytic effects of $PGF_{2\alpha}$ when administered by systemic routes (Douglas and Ginther, 1973, 1975; Oxender *et al.*, 1975). This may be explained, in part, by the high affinity of the equine $PGF_{2\alpha}$ receptor, which is about ten times greater than that of the receptors on the bovine corpus luteum (Kimball and Wyngarden, 1977), and would thus reduce the need for a local utero-ovarian $PGF_{2\alpha}$ concentrating mechanism.

Although $PGF_{2\alpha}$ is the primary luteolytic agent in ruminants, exposure of cultured bovine luteal cells to $PGF_{2\alpha}$ alone is not sufficient to inhibit progesterone production completely (Pate and Condon, 1984). Therefore, although $PGF_{2\alpha}$ has a central role in luteolysis, other mechanisms must be essential for complete luteal regression. A number of studies indicate that in mares, as in many other species, a number of agents, including other hormones such as oxytocin, oestrogen and progesterone, may be involved in triggering the release of $PGF_{2\alpha}$. In addition, the mechanism by which prostaglandin might initiate luteolysis is not clear, although it has been the subject of studies in several species giving a variety of results (Nett *et al.*, 1976; Niswender *et al.*, 1976; Fletcher and Niswender, 1982; Silvia *et al.*, 1984; Hoyer and Marion, 1989; Wiltbank *et al.*, 1989a, b; Behrman *et al.*, 1993).

If luteolysis involves an immune response, it would have to be suppressed during maternal recognition of pregnancy. Infiltration of lymphocytes and macrophages into the bovine (Lobel and Levy, 1968) and rabbit (Bagavandoss *et al.*, 1988; Bagavandoss *et al.*, 1990) corpus luteum of pregnancy does not occur to the same extent as in the corpus luteum of the normal oestrous cycle. In addition, MHC class II expression is significantly decreased on all cell populations comprising the corpus luteum of pregnant compared with non-pregnant cows (Fairchild Benyo *et al.*, 1991) and sheep (Kenny *et al.*, 1991).

The purpose of this study was to investigate immune cell populations and expression of MHC class II in the equine corpus luteum throughout the oestrous cycle, in early pregnancy and after administration of exogenous $PGF_{2\alpha}$.

Materials and Methods

Animals

Genitally normal healthy pony mares weighing 250–350 kg and aged between 3 and 15 years were used in this study.

The mares showed normal patterns of oestrus and ovulation throughout the spring and summer. They were examined by transrectal ultrasonography throughout the oestrous cycle to monitor follicular growth. During oestrus, the mares were scanned once a day until ovulation occurred (day of ovulation = day 0). The mares subsequently underwent unilateral ovariectomy by colpotomy (Colbern and Reagan, 1987) at different stages of dioestrus: early (days 2–4), mid- (days 7–10) and late dioestrus. The last group of mares was further divided into pre-luteolytic (days 12–14) and post-luteolytic (day 16+). Another group of mares was ovariectomized during the mid-luteal phase, 24 h after administration of exogenous $PGF_{2\alpha}$ analogue (1 ml Estrumate i.m. (equivalent to 263 µg cloprostenol); Mallinckrodt Veterinary Ltd, Uxbridge). The last group of mares in the study were mated by artificial insemination, diagnosed as pregnant by ultrasound scan and subsequently monitored to confirm continuing pregnancy up to and at the time of surgery. The ovary containing the corpus luteum was removed between day 20 and day 50 of pregnancy. Each group consisted of five to eleven mares.

All surgery was performed under standing sedation and analgesia using acepromazine (0.05 mg kg^{-1} i.m.; C-Vet Ltd, Bury St Edmunds), romifidine (0.05 mg kg^{-1} i.v.; Sedivet, Boehringer Ingelheim Ltd, Bracknell), butorphanol (0.05 mg kg^{-1} i.v.; Torbugesic, Willow Francis, Crawley) and flunixin meglumine (1.1 mg kg^{-1} i.v.; Finadyne, Schering-Plough Animal Health, Welwyn Garden City). Tetanus prophylaxis was administered before surgery and all mares received in-feed trimethoprim and sulphadiazine (30 mg kg^{-1} day^{-1}; Uniprim, Cheminex Laboratories Ltd, Corby) for 4 days after surgery. Blood samples were collected from all mares by jugular venepuncture into an evacuated heparinized tube (Becton Dickinson UK Ltd, Cowley, Oxford) for progesterone analysis for 2–3 days before and immediately before surgery, to assess corpus luteum function. Blood samples were centrifuged at 2000 *g* for 15 min at 4°C. Plasma was stored at –20°C before the assays were perfomed.

Processing of samples

Immediately after surgery, the corpus luteum was dissected out of the ovarian tissue. Samples for immunohistochemistry were placed in OCT compound (Miles Inc., Elkhart, IN), snap frozen in dry ice and isopentane, and stored at –70°C. Fresh tissue was processed immediately for analysis by flow cytometry.

One piece of corpus luteum was fixed for 24 h in 4% (w/v) paraformaldehyde. These samples were used for histochemical staining.

Progesterone assay

Progesterone concentrations were determined in unextracted plasma using a validated technique (Corrie *et al.*, 1981) modified by Law *et al.* (1992). Standards were prepared in plasma from an ovariectomized mare. The main cross-reactivities of the antiserum were with 5-pregnane-3,20 dione (9.5%), 11-deoxycorticosterone (6.2%), and 17-hydroxy-progesterone (3.4%). The limit of detection of the standard curve was 0.5 ng ml^{-1}, and the intra- and interassay coefficients of variation were 9 and 12.6%, respectively (Watson *et al.*, 1995).

Immunohistochemistry

Cryostat sections (6 µm thick) were cut from each sample, placed on gelatin-coated (Biobond; British Biocell International, Cardiff) slides and air-dried for 2 h before they were fixed for 5 min in acetone at 4°C. Sections were washed in PBS (pH 7.3) and stained according to a modified avidin–biotin complex (ABC) staining method (Hsu *et al.*, 1981) using a Vectastain Elite ABC Kit (Vector Laboratories, Peterborough) and primary monoclonal antibodies (mAbs) against immune cell specific antigens. mAbs to CD4 (HB 61A), CD8 (HT 14A) and CD5 (HB 19A) were purchased from VMRD (Washington State University) and a mAb to B cells (MAC 292) was obtained from the Animal Health Trust (Newmarket). The specificities of these antibodies were reported by Kydd *et al.* (1994). Three anti-MHC class II mAbs were used in the study. VPM 54 (Department of Veterinary Pathology, University of Edinburgh) and TH14B (VMRD, Washington State University) are specific for the ruminant MHC class II DR locus, and VPM 36 (Department of Veterinary Pathology, University of Edinburgh) is DQ specific (Dutia *et al.*, 1995). All these mAbs crossreact with the equine antigen as there is strong interspecies conservation of the MHC antigen (Hopkins *et al.*, 1986). After initial investigations in which all three antibodies produced almost identical staining patterns, VPM 36 and 54 were used throughout the rest of the study.

Endogenous peroxidase staining was blocked by incubating the sections with 5 U glucose oxidase ml^{-1} in 10 mmol *B*-D glucose l^{-1}, 1 mmol sodium azide l^{-1} and 0.1 mol PBS l^{-1} (pH 7.3) for 50 min at 37°C. The sections were treated with 1.5% normal horse serum for 15 min at room temperature, followed by overnight incubation at 4°C with the primary antibodies. The sections were washed for 10 min with PBS before they were incubated with the secondary biotinylated antibody for 30 min at room temperature. The avidin–biotin complex reagent was added after further washing, and the sections again incubated for 30 min at room temperature. Chromagen (3-amino-9-ethylcarbazole, AEC) was added as the final substrate and a red reaction product was visible after 7–8 min. Sections were then washed in tap water, counter-stained with Meyer's haematoxylin and mounted using an aqueous mounting agent (Immumount; Shandon, Pittsburgh, PA). Negative control sections in which serum was substituted for the primary antibody were included, while sections of equine lymph node were used as positive controls throughout.

Histochemistry

Frozen sections were also stained for non-specific esterases (Hudson and Hay, 1989) to aid identification of

macrophages. Carbol-chromotrope staining (Lendrum, 1944) was carried out on sections from samples fixed in paraffin for detection of eosinophils. Fixed sections were also stained with haematoxylin and eosin to confirm that cell morphology had been adequately preserved.

Cell counts

Preliminary studies indicated that the immune cells were more numerous in the trabeculae of the corpus luteum than among the luteal cells. Immune cells were also found in greater numbers in the capsule of the corpus luteum. For this reason, the capsule and associated fibrous tissue were avoided when examining the samples. Only extravascular positively stained cells present in the viewing field were counted. Five randomly selected fields were examined at low power: representative fields were selected from the outer, middle and inner areas of each corpus luteum. The mean of the five fields was used as the final figure.

Flow cytometry

Samples were divided into three groups: mid- (days 6–8), late pre-luteolytic (days 12–14) and late post-luteolytic (days 16–17) luteal phase. Luteal tissue was finely minced and resuspended in 30 ml Hank's balanced salt solution (Ca^{2+} and Mg^{2+} free) buffered with 20 mmol Hepes (buffered HBSS; Flow Laboratories, Irvine). Cells were mechanically dissociated from the tissue by gentle inversion 10–15 times. The suspension was passed through a 100 μm stainless steel mesh and the cells were collected by centrifugation (400 *g*, 10 min) and resuspended in buffered HBSS. The cells were washed twice in fresh ice-cold FACS buffer (1% bovine serum albumin and 0.1% (w/v) azide in PBS) and counted in a haemocytometer. A cytospin preparation of each tissue sample was stained with Diff-Quick™ (Baxter, Thetford) to evaluate the cell population obtained by the dissociation process. Cells from individual corpora lutea were processed for indirect immunofluorescence.

Approximately 1×10^5 viable luteal cells per tube were incubated for 30 min at 4°C with optimal dilutions of each of three primary mAbs for MHC class II antigen: TH14B (VMRD Inc., Pullman, WA) and VPM 36 and 54 tissue culture supernatants (Department of Veterinary Pathology, University of Edinburgh). Controls included cells incubated with 1:500 normal mouse serum substituted for the primary antibody to monitor possible non-specific binding of antibody, and cells incubated with PBS instead of antibody. Cells were washed twice with FACS buffer and visualized with fluorescein isothiocyanate (FITC)-conjugated rabbit anti-mouse immunoglobulin antiserum (Dako, High Wycombe) diluted 1:250. Cells were again washed twice in FACS buffer and fixed in 1% (w/v) paraformaldehyde (BDH, Poole) and maintained at 4°C in the dark until the analysis was performed within 6 days of labelling.

A minimum of 5000 cells from each sample was analysed using a fluorescence-activated cell analyser (FACScan; Becton Dickinson, Los Angeles, CA). Cells with different physical characteristics (that is, size and complexity) were analysed separately by gating using forward scatter (FSC: cell size) and side scatter (SSC: cell complexity) parameters. Gating of the scatter plots after flow cytometry permitted isolation of cell populations of interest and exclusion of debris and red blood cells. Equine luteal cells are relatively large, measuring 26–36 μm depending on the stage of the oestrous cycle. As MHC class II expression by these cells was of interest, a gate was set that encompassed the largest cells on the plot (Fig. 1, area A) and excluded debris, red blood cells and inflammatory cells (Fig. 1, area B). Early corpora lutea (days 2–4) were excluded from flow cytometry due to the large proportion of red blood cells present in these samples. The percentage of positively labelled cells was calculated by subtracting signals from non-specifically labelled cells.

Statistical analysis

Immunohistochemistry results were analysed using the Kruskal–Wallis test for non-parametric data. When differences between samples were present they were then analysed using the Mann–Whitney U test. Flow cytometry results were analysed using one-way ANOVA. A P value < 0.05 was considered to be significant. All statistical analyses were performed using Minitab software (Pennsylvania State University, PA).

Results

Progesterone assay

Progesterone concentrations were used to determine corpus luteum activity in the mares at the time of surgery. Progesterone concentrations decrease rapidly at functional luteolysis before structural luteolysis and physical regression, and are therefore a very sensitive indicator of alterations in luteal performance. Luteolysis was considered to have occurred when plasma progesterone concentrations were < 2 ng ml^{-1}. All the animals in the days 2–4, days 7–10 and days 12–14 groups had progesterone concentrations consistent with the presence of a functional corpus luteum before and at surgery. Mares in the days 16–17 group had progesterone concentrations that indicated that functional luteolysis had occurred, as did those animals that had been administered $PGF_{2\alpha}$.

Immune cell detection

At all stages of dioestrus, lymphocytes were more numerous in the trabeculae of the corpus luteum than among the luteal cells (Fig. 2). CD4+ and CD8+ cells showed similar distribution patterns and were present in approximately equal numbers. CD4+ cells were present in significantly greater numbers in the samples from days 16–17 than in the tissues from days 2–4 ($P < 0.01$) or days 7–10 ($P < 0.05$) (Fig. 3). Visual examination revealed an apparent increase in the

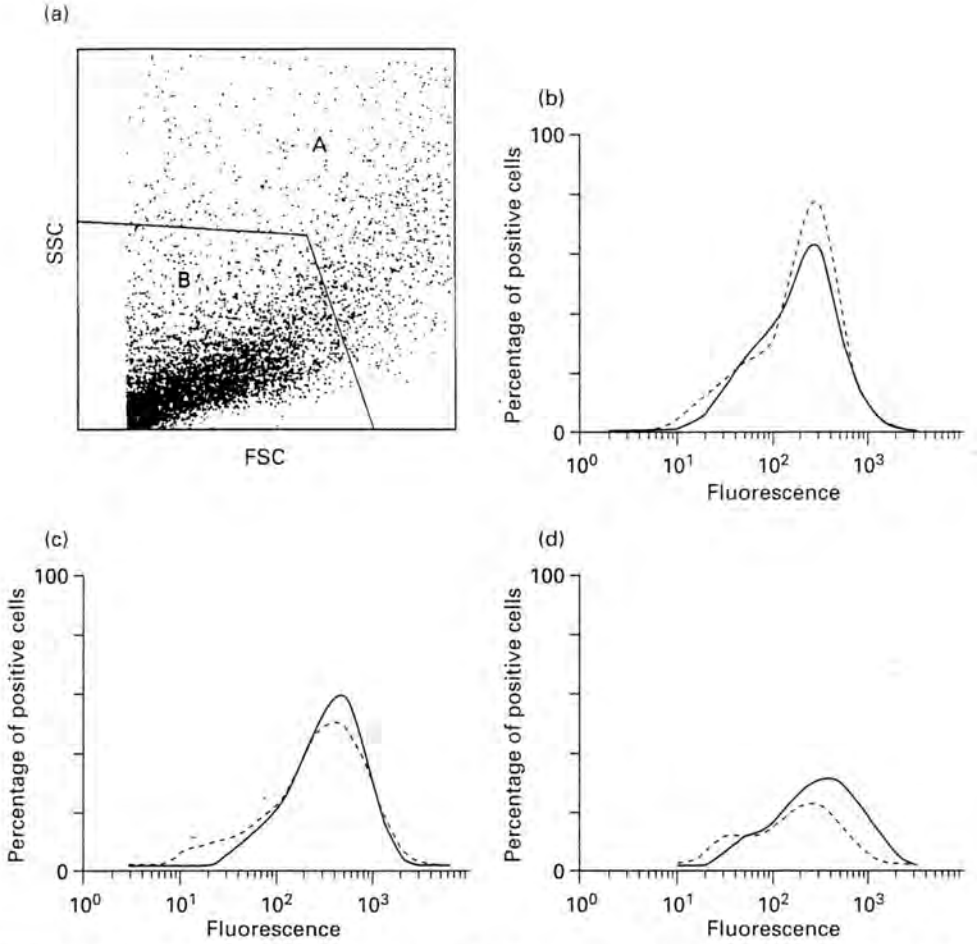

Fig. 1. Flow cytometry results from equine luteal cell preparations and suspensions. (a) Dot-plot showing the gate setting used forward scatter (FSC: cell size) and side scatter (SSC: cell complexity) parameters for analysis of large luteal cells (area A) and exclusion of small cells and cell debris (area B). Major histocompatibility (MHC) class II expression by (b) day 7, (c) day 12 and (d) day 16 corpora lutea. Broken lines represent blanks (cells incubated with normal mouse serum instead of monoclonal antibody). Solid lines represent MHC class II expression.

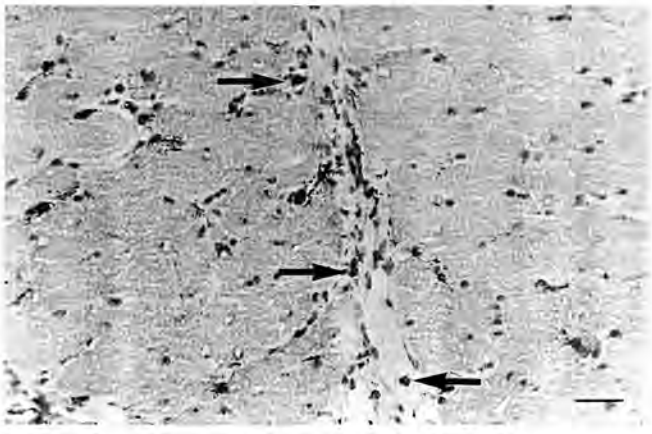

Fig. 2. CD8 (HT14A)-positive cells (arrows) in the trabeculae of a day 12 equine corpus luteum. Scale bar represents 15 μm.

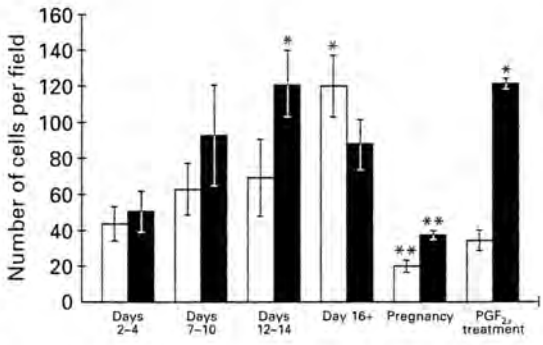

Fig. 3. Number of CD4+ (□) and CD8+ (■) T cells in the equine corpus luteum during the oestrous cycle, early pregnancy and after administration of $PGF_{2\alpha}$. Values are mean ± SEM. *, indicates values significantly higher ($P < 0.05$) than all other stages examined (but not higher than the other value at the same stage); **, indicates values significantly lower ($P < 0.05$) than all other stages examined.

number of cells in the trabeculae during the late luteal phase along with a migration of cells among the luteal cells (Fig. 4). CD8+ cells were present in significantly greater numbers at days 12–14 than at days 2–4 ($P < 0.05$). CD5+ cells were present in small numbers throughout the cycle and there were no significant differences among the groups.

Populations of CD4+ cells were significantly smaller in early pregnancy than at any stage of the oestrous cycle

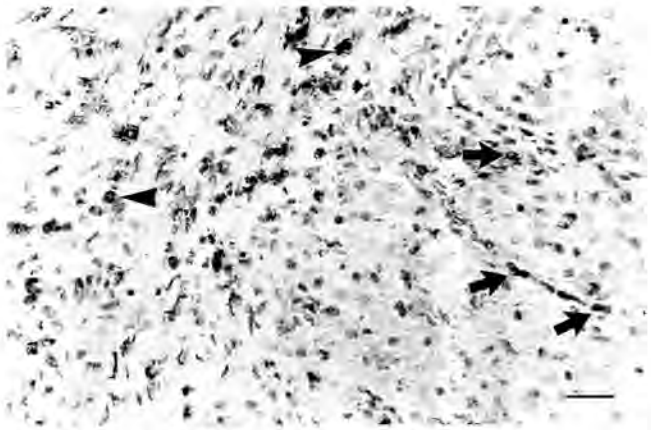

Fig. 4. T lymphocytes (HB16A) in a large trabecula (arrowheads) of a day 16 equine corpus luteum and movement of cells (arrows) into luteal tissue. Scale bar represents 15 μm.

($P < 0.01$, except days 2–4: $P < 0.05$) (Fig. 3). Corpora lutea from pregnant mares also contained significantly smaller numbers of CD8+ cells than at days 12–14 or days 16–17 of the oestrous cycle ($P < 0.01$) (Fig. 3). There was no significant difference in the number of CD5+ cells in early pregnancy compared with any stage of the oestrous cycle.

B cells were present in very small numbers at all stages of the oestrous cycle; the average number per field examined was between zero and three cells. However, the number of cells was significantly greater in corpora lutea from the early (days 2–4; $P < 0.05$) and late (days 16–17; $P < 0.01$) phases than the mid-luteal phase (days 7–10). The number of B cells was significantly lower in pregnancy compared with early luteal (days 2–4) samples ($P < 0.05$).

Cells positive for non-specific esterase staining (macrophages) were present in significantly greater numbers in post-luteolytic samples (days 16–17) than at any other stage of the oestrous cycle or early pregnancy ($P < 0.05$) (Fig. 5). In addition, there was a significant increase in positive cells between samples from days 7–10 and those from days 12–14 ($P < 0.05$).

The number of MHC class II-positive cells was significantly increased in samples from days 16–17 compared with tissues from days 7–10 (Fig. 6). One of the mAbs (VPM 54) also showed a significant increase in expression between samples from days 2–4 and days 16–17 ($P < 0.05$). MHC class II expression decreased in early pregnancy. The number of cells expressing MHC class II was significantly smaller in pregnancy than at days 12–14 ($P < 0.05$) and days 16–17 ($P < 0.01$) of the oestrous cycle.

Eosinophils were present in very small numbers throughout the oestrous cycle and showed no significant differences in numbers during the period studied. A small number of eosinophils was also present in early pregnancy; this number was significantly smaller than at days 12–14 of the oestrous cycle ($P < 0.05$).

After $PGF_{2\alpha}$ administration, the number of CD4+ cells was significantly smaller than after natural luteolysis ($P < 0.01$), whereas the number of CD8+ cells was not significantly altered (Fig. 3). The number of CD5+ cells was also unchanged, whereas the number of B cells was significantly smaller than in

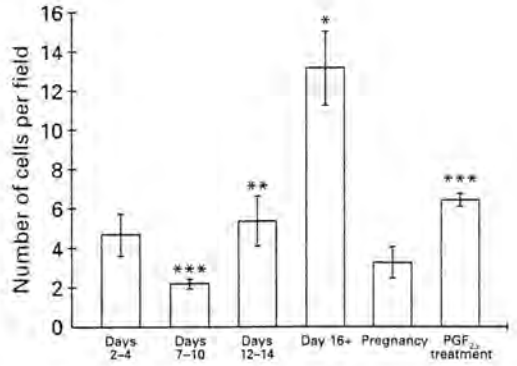

Fig. 5. Number of macrophages (cells positive for non-specific esterase staining) in the equine corpus luteum during the oestrous cycle, early pregnancy and after administration of $PGF_{2\alpha}$. Values are mean ± SEM. *, indicates value significantly higher ($P < 0.05$) than all other stages examined; ** and ***, indicate values that are significantly different from each other ($P < 0.05$).

samples from days 16–17 ($P < 0.05$). There was no difference in MHC class II expression after $PGF_{2\alpha}$ administration compared with after natural luteolysis for both mAbs used (Fig. 6) but there was significantly greater expression ($P < 0.05$) than in samples from days 7–10 and pregnant mares. Cells positive for non-specific esterase staining were present in significantly smaller numbers ($P < 0.05$) compared with samples from days 16–17, but in significantly greater numbers than in samples from days 7–10 (Fig. 5).

Eosinophils were present in significantly greater numbers ($P < 0.05$) in tissues from prostaglandin-treated mares than in tissues from mares that had undergone natural luteolysis.

Flow cytometry

Negligible MHC class II expression by equine luteal cells was detected with all three mAbs at all stages of the oestrous

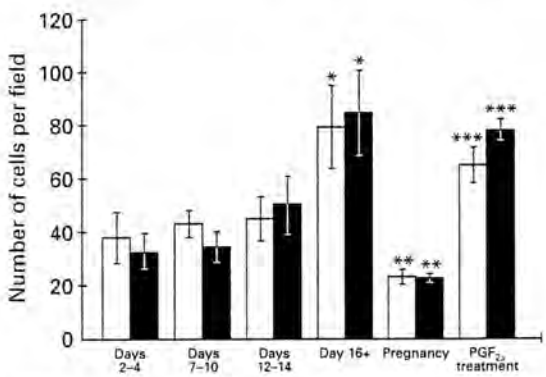

Fig. 6. MHC class II expression in the equine corpus luteum during the oestrous cycle, early pregnancy and after administration of $PGF_{2\alpha}$ using two different monoclonal antibodies: VPM 36 (□) and VPM 54 (■). Values are mean ± SEM. *, indicates values significantly higher ($P < 0.05$) at days 7–10 and early pregnancy; **, indicates values significantly lower ($P < 0.05$) than at days 12–14 and 16–17; ***, indicates values significantly higher ($P < 0.05$) than days 7–10 and pregnancy.

cycle examined. In addition, there were no significant differences in expression among any of the time periods analysed (Fig. 1).

Discussion

Different inflammatory cell populations have been observed in the ovaries of a number of species throughout the oestrous cycle. Studies on the local interactions occurring between immune and reproductive cells indicate a key role for leucocytes and their products in ovarian physiology. To date, there have been no reports on immune cell populations in the equine corpus luteum. The results of this study show that the immune cell populations in the equine corpus luteum vary during the oestrous cycle and after administration of $PGF_{2\alpha}$.

The macrophage is the best documented ovarian cell of lymphohaematopoietic origin and has been localized in the ovary of several species (Paavola, 1979; Lei *et al.*, 1991; Wang *et al.*, 1992; Brannstrom and Norman, 1993). Initially, macrophages and other leucocytes were thought to be involved in removing luteal cells by phagocytosis during structural regression. However, cells of the mononuclear phagocyte system possess considerable functional heterogeneity in tissues and body cavities. In addition to their phagocytic role, macrophages secrete a wide variety of products that are involved in connective tissue breakdown and vascular changes and are ovulatory mediators (Brannstrom *et al.*, 1994). They are also a rich source of multifunctional cytokines such as tumour necrosis factor α (TNF-α) and interleukin 1 (IL-1), both of which are capable of influencing various ovarian cell functions (Roby and Terranova, 1989; Nakamura *et al.*, 1990; Roby *et al.*, 1990; Zolti *et al.*, 1990; Hurwitz *et al.*, 1991; Veldhuis *et al.*, 1991).

In the present study, identification of macrophages was problematic. A number of antibodies were tested with highly variable results and marked differences among antibodies. Therefore, non-specific esterase staining was used for detection of macrophages in this study. Cellular esterases are ubiquitous and appear to represent a series of different enzymes acting upon select substrates. Under defined reaction conditions, it is possible to determine haematopoietic types of cell using specific esterase substrates. Under the conditions used in this study, the enzyme detected is found primarily in monocytes, macrophages and histiocytes, and is virtually undetectable in granulocytes. Significantly increased numbers of positive cells were present in post-luteolytic samples compared with every other stage of the cycle. However, there was also a significant increase in the number of positive cells between samples from days 7–10 and days 12–14. This may indicate an influx of macrophages into the equine corpus luteum before functional luteolysis, implying a possible role for macrophages and their array of secretory products in functional as well as structural luteolysis.

CD4+ and CD8+ cells were present in approximately equal numbers during dioestrus. The number of CD4+ cells increased significantly in the corpus luteum after luteolysis compared with early and mid-luteal samples. As this increase occurred after functional luteolysis, the influx may not be relevant to the regression of the corpus luteum. However, CD8+ cytotoxic T cells increased significantly before functional luteolysis (days 12–14), which indicates a role for these cells and their products in luteolysis. Furthermore, in the peripheral blood of horses, the ratio of CD4+:CD8+ T cells is > 2:1(Lunn *et al.*, 1991), indicating that there is selective infiltration of CD8+ lymphocytes into the equine corpus luteum. A similar observation has been noted in the rat ovary during the periovulatory period (Brannstrom *et al.*, 1993), while in cows, CD8+ cells appear to be involved in functional luteolysis (Ndikum-Moffor *et al.*, 1994). Thus, it seems that these cells may have a physiological role in control of ovarian function. Activated T lymphocytes are a rich source of a variety of cytokines such as IL-2 and interferon γ (IFN-γ), both of which affect steroidogenesis of human granulosa-lutein cells (Wang *et al.*, 1991, 1992). IFN-γ also alters bovine luteal cell prostaglandin production and progesterone synthesis (Fairchild and Pate, 1991), which indicates a role in luteolysis, and can also have direct cytotoxic effects on cells especially when present in combination with some other cytokines (Campbell *et al.*, 1988; Fairchild and Pate, 1991; Benyo and Pate, 1992). Such properties, when taken in conjunction with cyclic variations in the number of cells, indicate a physiological role for T cells in cyclic ovarian activity.

In the present study, three different T-lymphocyte markers were used. CD4+ helper T cells and CD8+ cytotoxic killer T cells are two mutually exclusive subpopulations of T lymphocytes in horses (Lunn *et al.*, 1991; Kydd *et al.*, 1994), the numbers of which, when added together, equal those of the CD5+ cells, a pan-T-cell marker (Kydd *et al.*, 1994). This was not the case in the present study in which CD5+ cells were present only in very small numbers and were always greatly outnumbered by other T cells. Research in sheep revealed a similar discrepancy (Hopkins and Dutia, 1990), and it appears that the CD5 molecule is not present at cellular activation. This proposal would explain other reports in which CD4+ and CD8+ cells greatly outnumbered CD5+ cells (Gorrell *et al.*, 1988, Meeusen *et al.*, 1988). In the present study, the number of CD5+ cells in the equine corpus luteum did not vary significantly throughout the oestrous cycle or in early pregnancy.

B lymphocytes were present in very small numbers throughout the oestrous cycle. Studies of the human corpus luteum failed to detect any B cells during the menstrual cycle (Wang *et al.*, 1992; Brannstrom *et al.*, 1994). Reports in a number of other species also fail to report the presence of B cells (Standaert *et al.*, 1991; Petrovska *et al.*, 1992; Brannstrom *et al.*, 1994). B cells are antibody-producing cells that are active in humoral immune responses, a role which is probably not relevant to the function of the corpus luteum.

MHC class II expressing cells detected by immunohistochemistry were present in significantly greater numbers in post-luteolytic samples (days 16–17) than in mid-luteal tissue. An increase in number was noted in samples from days 12–14, although the difference was not significant. This increase probably reflects the increased number of immune cells present as the corpus luteum undergoes structural luteolysis and the phagocytic cells move in. Expression of MHC class II by equine large steroidogenic

luteal cells was not detected in this study, either by immunohistochemistry or flow cytometry. This finding is in contrast to studies in cows (Fairchild Benyo *et al.*, 1991) and sheep (Kenny *et al.*, 1991) which reported MHC class II expression by luteal cells towards the end of the luteal cycle and after $PGF_{2\alpha}$-induced luteolysis (Fairchild Benyo *et al.*, 1991). However, Anderson (1997) also failed to detect MHC class II expression in the bovine corpus luteum.

Eosinophil infiltration into the corpus luteum during luteolysis has been observed in a number of species, particularly sheep (Nett *et al.*, 1976; Murdoch *et al.*, 1988) and pigs (Standaert *et al.*, 1991). A role for these cells in initiation of luteal regression has been indicated in pigs (Standaert *et al.*, 1991). In the present study, eosinophils were present in the equine corpus luteum in very small numbers throughout the oestrous cycle, and showed no significant increase at luteolysis.

If immune cells are involved in luteolysis, the immune response would have to be suppressed during maternal recognition of pregnancy. The findings in the present study are in agreement with studies in cows (Lobel and Levy, 1968) and rabbits (Bagavandoss *et al.*, 1988) in which a decreased infiltration of lymphocytes and macrophages into the corpus luteum of pregnancy was observed. Significantly smaller numbers of both CD4+ and CD8+ cells were observed in early pregnancy than during the oestrous cycle. The number of macrophages also decreased significantly in early pregnancy. MHC class II expression was reduced in the equine corpus luteum in early pregnancy, but this may simply reflect the decreased number of T lymphocytes and macrophages present in the tissue. In contrast, MHC class II expression is significantly decreased in both luteal and non-luteal cell populations comprising the corpus luteum of pregnant compared with non-pregnant cows (Fairchild Benyo *et al.*, 1991) and sheep (Kenny *et al.*, 1991). The signal for suppression of MHC class II expression in these species is not known, but may be related to the trophoblast protein IFN-τ (Pate, 1995). No equivalent conceptus-derived protein has been identified in mares.

In veterinary practice, the exogenous administration of $PGF_{2\alpha}$ is commonly used to induce luteolysis artificially in mares. The dose used is the same as that used in the present study, which caused some interesting changes in cell populations in the corpus luteum. Plasma progesterone concentrations indicated that the mares had undergone functional luteolysis by the time of ovariectomy. The number of CD4+ cells was significantly smaller than after naturally occurring functional luteolysis, whereas there was no significant difference in populations of CD8+ cells. MHC class II expression was also not significantly different from that after natural luteolysis.

The non-specific esterase staining results showed significantly fewer positive cells, considered to represent macrophages, after prostaglandin treatment compared with samples from days 16–17. It is possible that the more rapid regression of the corpus luteum after artificially induced luteolysis did not allow sufficient time for the macrophages and CD4+ cells to infiltrate the tissue to the same extent as observed after natural luteolysis. In the present study, samples were taken from 1 to 3 days after natural functional luteolysis, as determined by progesterone concentrations, compared with 24 h after prostaglandin administration. Alternatively, induced luteolysis may not follow the same series of events observed in natural luteolysis, and substances that attract macrophages may be missing from the process. The pharmacological dose of prostaglandin used to induce luteolysis artificially is many orders of magnitude greater than normal physiological concentrations of endogenous prostaglandin in the corpus luteum at luteolysis, and this may also alter the normal order of events. $PGF_{2\alpha}$ has marked vasoconstrictive actions that are thought to be responsible for some of the morphological changes in the corpus luteum at luteolysis (Nett *et al.*, 1976; Stacy *et al.*, 1976). The excessive dose of prostaglandin used to induce luteolysis may also exaggerate this effect. In addition, the vasoconstrictive effects of $PGF_{2\alpha}$ may affect the infiltration of inflammatory cells into the corpus luteum.

In the present study, eosinophils were present in the equine corpus luteum in very small numbers throughout the oestrous cycle, and showed no significant increase at luteolysis. In contrast, after $PGF_{2\alpha}$ administration, the number of eosinophils increased to significantly greater amount than after natural luteolysis. This indicates that exogenous prostaglandin can attract eosinophils into the equine corpus luteum, either directly or through changes induced in the luteal tissue.

In conclusion, the results of the present study indicate that various immune cells, particularly lymphocytes, differentially migrate into, and out of, the equine corpus luteum during the oestrous cycle, early pregnancy and after administration of $PGF_{2\alpha}$. The temporal nature of this migration indicates involvement of certain populations of these cells in modulation of luteal activity. Further studies are required to identify specific products of these cells that are present throughout the life of the corpus luteum to clarify the role of immune cells within this tissue.

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Chemoattractant properties of conditioned medium from equine *corpora lutea* collected at various stages of the oestrous cycle

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Keywords: horse; *corpus luteum*; chemotaxis

Summary

This study investigated the chemotactic activity of equine CL at different stages of the oestrous cycle. The purpose of this was to ascertain whether luteal tissue itself contributes to the massive influx of leucocytes around the time of natural and induced luteal regression. *Corpora lutea* were collected at different stages of dioestrus and after treatment with $PGF_{2\alpha}$. Culture medium harvested after incubation of luteal tissue for 20 h was chemotactic for both polymorphonuclear and mononuclear cells in late dioestrus (before functional regression) as well as after natural and induced luteal regression. By contrast, midluteal tissue showed no chemotactic activity. This is the first report of the ability of equine luteal tissue actively to recruit inflammatory cells *in vitro* and supports our earlier findings that this infiltration starts prior to functional luteolysis. We hypothesise that this early influx of inflammatory cells may play an active role in luteal regression. Further research is needed to identify the specific chemotactic factor(s).

Introduction

White blood cells within follicles and *corpora lutea* (CL) are thought to be involved in follicular atresia, ovulation and luteolysis (Espey 1980; Murdoch et al. 1988; Adashi 1990; Mori 1990). We have shown previously that immune cells migrate into the equine CL prior to functional luteolysis, and after administration of $PGF_{2\alpha}$ (Lawler *et al.* 1999; Al-zi'abi *et al.* 2002). The timing of this migration suggests that certain populations of these cells may be involved in modulation of luteolysis. There is very little information regarding chemotactic factors in ovarian tissues that might be involved in modulating leucocyte migration into the CL. Follicular fluid from cattle, women and horses has been shown to possess chemotactic activity (Seow *et al.* 1988; Watson *et al.* 1991; Sirotkin and Luck 1995). Leucocyte attractant activity has also been identified in medium conditioned by luteal and follicular tissue from cattle and sheep (Murdoch 1987; Murdoch and McCormick 1989, 1991; Sirotkin and Luck 1995) and 2 peptides possessing significant chemotactic activity have been sequenced from follicle-conditioned medium (Murdoch and McCormick 1992).

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There is no information, however, on production of chemoattractants by the CL at different stages of the oestrous cycle or after administration of $PGF_{2\alpha}$. Several potential chemotactic factors are present in the ovary. These include C5a (Sedgwick *et al.* 1987; Murdoch and McCormick 1991) and the potent chemoattractant LTB4, which is present in high concentrations in equine luteal tissue (Watson 1990). Studies have suggested that some of the chemotactic substances in the ruminant CL are collagen breakdown products as well as collagenase (Murdoch and McCormick 1993; Sirotkin and Luck 1995). In addition, cytokines, such as interleukin-1 (IL-1), tumour necrosis factor-α (TNF-α), interleukin-8 (IL-8) and granulocyte-macrophage colony-stimulating factor (CSF), are important chemoattractants in other species and are present in the ovary (Norman and Brannstrom 1994), where they could be produced by leucocytes resident in the ovary, or by the ovarian cells themselves.

In the present study, the chemotactic activity of equine CL at different stages of the cycle was investigated to ascertain whether luteal tissue itself contributes to the massive influx of leucocytes around the time of natural and induced luteal regression.

Materials and methods

The ovaries of 11 mixed breed mares weighing 250–350 kg, age 3–15 years, were scanned daily by transrectal ultrasonography through oestrus. The day of ovulation was designated as Day 0. *Corpora lutea* were obtained from mares by ovariectomy (Lawler *et al.* 1999) at different stages of the luteal phase (Days 8–10, n = 3; 13–14, n = 2; 16–17, n = 3) and 24 h after administration of a luteolytic dose of $PGF_{2\alpha}$ to mares in midluteal phase (Days 8–10; n = 3).

Tissue preparation

The CL were dissected free from adherent tissue and cut into pieces of appproximately 1 mm^3, which were washed 3 times in medium (DMEM/Hams F-12 in a 1:1 mixture) supplemented with penicillin/streptomycin (1 unit/ml and 1 μg/ml, respectively, bovine serum albumin (Fraction V; 1 mg/ml), insulin (1 μg/ml), transferrin (0.55 mg/ml), selenium (as sodium selenite, 6.7 ng/ml) and ascorbic acid (50 μg/ml). Tissue was then incubated in the same medium in 24 well plates for 20 h at 37°C in a humidified atmosphere of 5% CO_2. Three pieces of tissue,

representative of the outer, inner and intermediate areas of the CL, totalling 75–100 mg weight, were incubated per well. Ovarian stromal tissue was also incubated in pieces of similar size. Medium incubated without tissue was used as a negative control. All incubations were carried out in triplicate, and medium was collected at the end of the incubations and stored at -20°C until the migration assays were performed.

Leucocyte preparation

Blood was drawn from healthy mature horses by jugular venipuncture into a vessel containing sodium citrate (1 ml 3.8%/10 ml blood). Leucocytes were then isolated by a modification of the method of Haslett *et al.* (1985) using discontinuous hypotonic plasma/Percoll gradients.

Whole blood was allowed to sediment by gravity for 30 min at room temperature. The upper leucocyte-rich plasma layer was aspirated and centrifuged at 380 *g* for 6 min. The platelet-rich plasma supernatant was decanted and centrifuged at 2400 *g* for 20 min to prepare platelet-poor plasma (PPP). The initial leucocyte-rich pellet was resuspended in 2 ml PPP in a 15 ml polystyrene tube and underlayed with 42% and 51% Percoll/PPP solutions prior to centrifugation at 255 *g* for 12 min. Polymorphonuclear (PMN) and mononuclear (MN) cell preparations were harvested from the 42/51% Percoll interface and the upper PPP/Percoll interface, respectively. The very small number of eosinophils present (mean ± s.e.m. 2.5 ± 0.26% of total leucocyte yield) settled either in the 51% Percoll layer or with the erythrocyte pellet. Cells were washed sequentially in PPP, phosphate buffered saline (PBS) without Ca^{2+} and Mg^{2+}, containing 0.1% BSA, and PBS prior to resuspension in PBS. Cell counts were performed prior to final resuspension to produce cell preparations of approximately 1 x 10^8 cells/ml. All the procedures outlined were performed at room temperature. Isolated cell populations were assessed using cytocentrifuge preparations stained with Diff-Quik[1] and cell viability was determined by trypan blue exclusion.

Leucocyte migration assay

The migration of leucocytes in response to the conditioned media was assessed by a modification of the method of Sirotkin and Luck (1995). A solution of Hanks balanced salt solution (HBSS) containing 0.5% agarose and 0.5% BSA, supplemented with HEPES (2.4 mg/ml), was pipetted onto microscope slides (approximately 4 ml/slide) allowed to solidify at room temperature and then maintained at 4°C for at least 1 h. Parallel rows of 3 circular wells, 2 mm diameter and 2 mm apart, were cut in the gel by means of needle tubing mounted in a Perspex frame (Dr M.R. Luck, University of Nottingham). The cut agarose plugs were removed with a hypodermic needle, and the slide was ready for use in the assay. All samples tested in the study were run in triplicate in at least 6 separate migration assays.

Tissue-conditioned media

Four μl PMN or MN cell suspension was pipetted into the central well, while negative control (medium incubated alone), experimental (luteal and ovarian tissue incubations) and positive control (standard pooled equine serum, stored at -20°C) samples were pipetted into flanking wells. Slides were incubated (PMN for 2 h, MN cells for 18 h) in a humidified atmosphere of 95% air:5% CO_2 at 37°C. After incubation, cells were fixed to the slide by immersion in 10% buffered formalin for at least 1 h. The agarose was carefully removed by gently inverting the slide. Slides were air-dried and stained with Leishmann's stain prior to microscopic examination.

Progesterone assay

Blood samples were collected by jugular venipuncture into evacuated heparinised tubes immediately prior to surgery. Plasma was removed by centrifugation and stored at -20°C prior to assay. Progesterone concentrations were measured by radioimmunoassay as described previously (Watson *et al.* 2000). The limit of detection for this assay was 0.5 ng/ml, and the inter- and intra-assay coefficients of variation were 12.6 and 9.0%, respectively.

Interpretation of results and statistical analysis

Slides were examined microscopically using an eyepiece micrometer to assess leucocyte migration. The degree of cell migration towards experimental media was measured using an eyepiece graticule. The distance travelled was expressed as a percentage relative to that occurring towards the standard serum positive control and the negative control. Nondirectional/random movement towards the negative control was taken as 0% migration and that towards serum as 100%, with all tested solutions falling between these 2 measurements.

For each test solution, the mean percentage of positive control distance travelled for all the assays in which it was included was calculated. Results were analysed using the Kruskal-Wallis test for nonparametric data. Where present, differences between samples were then analysed using the Mann-Whitney Test. A P value of less than 0.05 was taken to be significant. All statistical analysis was performed using Minitab software[2].

Results

Leucocyte preparations

Polymorphonuclear cell preparations consistently contained more than 95% neutrophils, with the remainder of cells comprising small numbers of eosinophils, monocytes and lymphocytes.

The MN cell preparations were not as uniformly pure. Contaminating neutrophils were the biggest problem, accounting for 3–30% of the cell preparation. The remainder of the cell preparation consisted of monocytes and lymphocytes. Samples with high levels of neutrophil contamination were excluded from the study, unless the cell types were clearly distinguishable on microscopic examination of the chemotaxis assay slide.

Migration assays

Neither PMN nor MN cell preparations were attracted to any of the media samples which had been conditioned with Days 8–10 luteal tissue (Fig 1). Days 13–14 luteal tissue incubation medium had chemoattractant activity for both PMN and MN cells which was significantly greater ($P<0.05$) than that of the Days 8–10 tissue. All the mares in the group had plasma progesterone levels consistent with the presence of a functional CL at the time of surgery (>1 ng/ml).

PMN and MN cells were also attracted to medium from

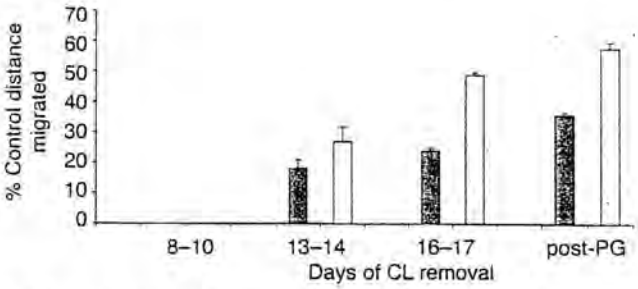

Fig 1: Mean migration of polymorphonuclear (filled bars) and mononuclear (open bars) cells towards medium conditioned with corpora lutea *collected at various stages of the oestrous cycle and after exogenous prostaglandin $PGF_{2\alpha}$ (data expressed as a percentage of distance travelled to the control serum sample).*

Days 16–17 luteal tissue. These tissue samples were confirmed as being postluteolytic by plasma progesterone assay of samples taken just prior to surgery (<1 ng/ml). For both cell populations, attractant activity of Days 16–17 tissue was significantly greater than Days 8–10 tissue ($P<0.05$), and tended to be greater than Days 13–14 samples ($P<0.10$).

Tissue samples taken 24 h after $PGF_{2\alpha}$ administration possessed the greatest chemoattractant activity. For PMN cells, attractant activity of PG-treated tissue was significantly greater ($P<0.005$) than Days 8–10 samples and Days 13–14 samples and tended to be greater than the Days 16–17 samples, but this was not significant. Similarly, tissue collected after PG treatment was significantly more attractive for MN cells than Days 8–10 samples ($P<0.001$) and Days 13–14 samples ($P<0.05$), and again tended to be greater than the Days 16–17 samples, but this was not significant. There was no indication that medium conditioned with ovarian stromal tissue collected at any of the stages of the cycle induced directional leucocyte migration.

Discussion

In the present study, we have shown for the first time that there is significant production of chemotactic factors by equine luteal tissue in the period before and after natural luteolysis and also after administration of $PGF_{2\alpha}$. We suggest that this could account for the leucocyte infiltration seen before and after luteolysis *in vivo* (Lawler *et al.* 1999). Cells were isolated using discontinuous hypotonic plasma-Percoll gradients. This method produces populations of cells which are minimally primed or activated and, therefore, better able to respond in chemotaxis assays (Haslett *et al.* 1985; Brazil *et al.* 1998). However, use of a variety of cell donors in the present study introduced a large source of variation in cell migration characteristics (Zinkl and Brown 1982; Haslett *et al.* 1985). For this reason, all test media were compared to the pooled standard serum run in the same assay.

Media incubated with Days 8–10 CL tissue was not chemotactic for either MN or PMN cell preparations. By Days 13–14, the tissue incubation media attracted both PMN and MN cells. These results indicate that the equine CL produces chemoattractant(s) for white blood cells prior to functional luteolysis taking place. The production of leucocyte chemoattractants prior to functional luteolysis is in agreement with previous results (Lawler *et al.* 1999) where we observed an increase in CD8+ T cells and macrophages in Days 12–14 luteal tissue from horses. The luteal chemoattractant may be a product of the luteal tissue, but it is also possible that the resident population of T lymphocytes and/or macrophages were producing cytokines, a number of which are known to be powerful chemoattractants.

Days 16–17 CL-conditioned media also attracted PMN and MN cells in agreement with *in vivo* data (Lawler *et al.* 1999). By this stage of the cycle the CL is undergoing structural regression, and it is possible that the presence of damaged cells and the inflammation associated with necrosis were involved in attracting phagocytically-active macrophages to help in the destruction and removal of luteal tissue. Furthermore, the equine CL may produce monocyte chemoattractant protein-1 (MCP-1) after functional luteolysis, as we have described recently in bovine CL (Penny *et al.* 1998). In the cow, this increase occurs prior to the increase in luteal macrophage numbers, and may indicate a role for MCP-1 in chemotaxis of monocytes into the CL during structural luteolysis.

Sirotkin and Luck (1995) examined the attractant properties of early (Days 1–2) CL tissue from cattle, and found that neutrophils and eosinophils were attracted to the tissue-conditioned media, while lymphocytes, monocytes and basophils were not. The earliest luteal tissue tested in our study was from Days 8–10 CL, and did not show any attractant activity for PMN or MN cell preparations. This difference may be due to the disparate ages of the tissues tested. The presence of inflammatory cells and chemoattractants for such cells in the newly formed CL may be a carryover from the inflammatory-like process of ovulation, with its associated release of a variety of inflammatory mediators and cytokines, many of which are known to have chemoattractant properties. Such substances may have been cleared from the luteal tissue at the time we obtained our earliest tissue samples, 8–10 days after ovulation.

Tissue collected after administration of $PGF_{2\alpha}$ to mares in the midphase tended to possess the greatest chemoattractant ability. Both PMN and MN cells migrated towards the tissue-conditioned media. The attractant activity tended to be greater than that of the Days 16–17 postluteolytic tissue samples. High numbers of lymphocytes, eosinophils and neutrophils have been observed in CL of mares treated with a luteolytic dose of $PGF_{2\alpha}$ (Lawler *et al.* 1999; Al-zi'abi *et al.* 2002). The pharmacological dose of $PGF_{2\alpha}$ used to artificially induce luteolysis in this study, and in veterinary practice, is many orders of magnitude greater than the normal physiological levels found in the equine CL, and may itself be responsible for the increased attractant activity of the $PGF_{2\alpha}$-treated tissue. However, $PGF_{2\alpha}$ has been reported not to be chemotactic for equine neutrophils (Watson *et al.* 1987), although the effect on MN cells was not assessed in that study. It is also possible that the high levels of prostaglandins cause alterations in surface antigens in the resident cells of the CL, perhaps exposing cellular antigens leading to antibody-mediated reactions involving complement (Murdoch *et al.* 1988), components of which are known to be powerful chemoattractants. In addition, cells damaged by the high PG levels may themselves release chemoattractants for inflammatory cells.

None of the ovarian stromal tissue samples, from any of the stages of the oestrous cycle examined or after $PGF_{2\alpha}$, expressed any chemoattractant properties. It appears, therefore, that the variations in attractant activity observed at different stages of the oestrous cycle are due to substances within the luteal tissue itself.

In conclusion, medium conditioned by equine luteal tissue taken at Days 13–14 and 16–17 of the oestrous cycle was chemotactic for equine leucocytes. Attraction of leucocytes by the CL prior to functional luteolysis may indicate an important role for these cells in demise of the CL. Postluteolytic tissue collected

after natural or induced regression exhibited strong attractant activity for mononuclear cells, and it is likely that these cells are involved in structural regression. Further studies are necessary to identify the chemical nature of the chemoattractants produced by the CL prior to and at luteolysis and the timing of their production relative to luteolysis.

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Research

Cell death during natural and induced luteal regression in mares

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In mares, little information is available on the type of cell death that occurs during natural and induced luteal regression. Corpora lutea were collected from mares in the early luteal phase, days 3–4 ($n = 4$); mid-luteal phase, day 10 ($n = 5$); early regression, day 14 ($n = 4$); late regression, day 17 ($n = 4$); and 12 and 36 h ($n = 3$ per group) after $PGF_{2\alpha}$ administration on day 10. Histological and ultrastructural sections were examined and TUNEL was used to detect DNA fragmentation. In early luteal regression, there were more pyknotic luteal cells and extracellular round dense bodies compared with the mid-luteal phase. By late regression, there was a significant decline ($P < 0.01$) in the number of round dense body clusters and a marked accumulation of lipid. Twelve and 36 h after $PGF_{2\alpha}$ administration, changes were similar to those seen in natural regression, but there was also a marked infiltration of neutrophils. Accumulation of lipid was not apparent until 36 h after $PGF_{2\alpha}$ administration. Ultrastructural examination revealed rarefaction and distortion of the mitochondrial cristae in most of the luteal cells by the mid-luteal phase. Luteal cells showed shrinkage, accumulation of lipid with foamy appearance, and disruption in both smooth endoplasmic reticulum and mitochondria during natural and induced regression. Some luteal cells showed fragmented or pyknotic chromatin characteristic of apoptosis. Other luteal cells showed crenation of the nuclear membrane and shrinkage of the nucleus, features not characteristic of apoptotic cell death. In late regression, capillaries were obstructed by swollen endothelial cells and round dense bodies. These results show that structural regression may be initiated as early as the mid-luteal phase, and is clearly visible by day 14 in natural regression and 12 h after induced regression. Apoptosis did appear to be involved in luteolysis in the equine corpus luteum, but non-apoptotic changes were also observed in some luteal cells during regression. Accumulation of lipid was a late feature of luteal regression.

Introduction

The equine corpus luteum is functional for about 14–15 days during the non-fertile cycle (Daels and Hughes, 1993). Luteal regression, characterized by a decrease in progesterone production (functional regression) and cellular demise of luteal tissue (structural regression), is thought to be brought about by secretion of uterine $PGF_{2\alpha}$ (Douglas and Ginther, 1975). However, the precise cellular mechanisms involved in luteolysis are not fully understood.

In general, cells die by three recognized mechanisms: apoptosis, necrosis (Kerr *et al.*, 1972) or terminal differentiation (Stenn, 1983). During necrosis, cells develop increased permeability, which leads to cellular swelling, non-selective DNA degradation and inflammation in the surrounding tissues. Apoptosis is a process whereby cells die in a controlled manner. Morphologically, apoptosis is characterized by shrinkage and condensation of chromatin (marginated chromatin) or fragmentation into multiple, small dense bodies. Cells may then break up into discrete membrane-bound structures containing variable amounts of condensed chromatin or cytoplasm, which are then ingested by macrophages or neighbouring cells, or are extruded into the lumen of the blood vessels (Kerr *et al.*, 1972; Wyllie *et al.*, 1980). Alternatively, cells may shrink into a single dense round mass with a densely basophilic pyknotic nucleus (Wyllie *et al.*, 1980). Terminal differentiation is another type of cell death, whereby certain types of cell, such as the outer squamous layers of skin and the granulosa cells next to the antrum of atretic ovarian follicles, undergo nuclear destruction or expulsion before the cessation of cellular function leads to cell death (Stenn, 1983; van Wezel *et al.*, 1999).

Most recent studies on luteal cell death have used 3′ end-labelling of the DNA fragments (TUNEL) to determine the type of cell death (Zheng *et al.*, 1994; Bacci *et al.*, 1996; Young *et al.*, 1997; McCormack *et al.*, 1998). In situations in which apoptosis is the major form of cell death in a tissue, this technique can assist in quantifying the degree of apoptosis (Negoescu *et al.*, 1998). However, when necrosis

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occurs alongside apoptosis, TUNEL may lack specificity. Therefore, the use of this technique alone to detect the exact type of cell death in the corpus luteum may be misleading (Fraser *et al.*, 1999). Definitive identification relies upon examination of the ultrastructure of cells (Wyllie *et al.*, 1980).

Apoptosis has been described in luteal regression using electrophoresis or TUNEL to detect DNA fragments in cattle (Juengel *et al.*, 1993; Zheng *et al.*, 1994), sheep (Kenny *et al.*, 1994), pigs (Bacci *et al.*, 1996), women (Shikone *et al.*, 1996; Yuan *et al.*, 1997), monkeys (Young *et al.*, 1997), golden hamsters (McCormack *et al.*, 1998) and rabbits (Dharmarajan *et al.*, 1994). However, another form of cell death was observed alongside apoptosis in the corpus luteum of women and monkeys (Fraser *et al.*, 1995; Shikone *et al.*, 1996).

In mares, the corpus luteum starts regressing functionally from day 10 to 12 onwards and, on day 16, the luteal cells decrease in size. By day 20, two types of cell degeneration were reported by histological examination: (i) pyknosis associated with shrunken nuclei and very condensed cytoplasm; and (ii) karyolysis characterized by lysis of chromatin (Van Niekerk *et al.*, 1975). In another study, ultrastructural examination revealed that pyknotic nuclei were present on day 15 (Levine *et al.*, 1979). Data from these studies were limited to cellular changes during natural luteolysis. There have been no reports on the types of cell death that take place during $PGF_{2\alpha}$-induced luteolysis in mares. While noting that forms of cell death other than apoptosis may occur during luteolysis (Shikone *et al.*, 1996; Fraser *et al.*, 1999), it is important to use techniques that can differentiate types of cell death to study changes that may be specific to mares. In the present study, biochemical and ultrastructural methods were used to investigate the point at which structural luteolysis occurs and the types of cell death involved in both natural and $PGF_{2\alpha}$-induced luteal regression.

Materials and Methods

Animals

Pony mares of mixed breeding, between 4 and 12 years of age and 250–450 kg body weight were used. The ovaries were examined each day during oestrus by transrectal ultrasonography to determine day of ovulation (day 0). Blood samples (10 ml) were collected by jugular venepuncture into evacuated heparinized tubes for 5 days before ovariectomy to establish progesterone concentrations. Samples were centrifuged at 2000 ***g*** for 15 min at 4°C, and the plasma was stored at –20°C before assay. The ovary containing the corpus luteum was removed via a colpotomy incision after appropriate sedation and analgesia (Lawler *et al.*, 1999).

Tissue and blood collection

Corpora lutea were obtained in early luteal phase, days 3–4 ($n = 4$); mid-luteal phase, day 10 ($n = 5$); early regression phase, day 14 ($n = 4$); late regression phase, day 17 ($n = 4$); and 12 and 36 h ($n = 3$ each) after intramuscular administration of the $PGF_{2\alpha}$ analogue, cloprostenol (Estrumate, 263 µg per 500 kg, Schering-Plough Animal Health Ltd, Uxbridge) on day 10 of the oestrous cycle. The ovaries were transferred on ice to the laboratory immediately after surgical removal. The ovarian cortex layer was removed, and the corpus luteum enucleated from the ovary and dissected free of connective tissue. The corpus luteum was weighed and then cut to give representative pieces of both the central and peripheral areas. The samples were fixed in freshly prepared 4% (w/v) paraformaldehyde in 0.1 mol PBS l^{-1} for 24 h at 4°C, then processed the following day and embedded in paraffin wax. Samples (1 mm × 1 mm) from both peripheral and central areas of the corpora lutea were fixed for 4 h in 3% (v/v) glutaraldehyde in 0.1 mol cacodylate l^{-1} buffer at 4°C and post-fixed with 2% (v/v) osmium tetroxide for 1 h at room temperature. The samples were then washed three times in 0.1 mol cacodylate buffer l^{-1} and embedded in resin for transmission electron microscopy (TEM). Remaining representative samples were frozen in an isopentane–dry ice slurry and stored at –70°C.

Histological examination

Paraffin wax sections were stained by conventional histological methods using haematoxylin and eosin. Morphological criteria were used (Kerr *et al.*, 1972; Wyllie *et al.*, 1980) to differentiate and quantify cells that were actively undergoing apoptosis at the time of fixation, from dense small round bodies that were considered to represent residual products of apoptosis (apoptotic bodies). Apoptotic cells were defined as cells with nuclei containing condensed chromatin that was: (i) marginated into delineated, densely staining masses aligned with the nuclear membrane (marginated chromatin); (ii) shrunken into a single, round regularly shaped, dense, homogeneously staining mass (pyknotic appearance); or (iii) fragmented into multiple homogeneously dense masses (multiple fragments) situated inside the cells. Apoptotic bodies were considered to be remnants of apoptotic cell death, and defined as discrete membrane-bound structures containing various amounts of condensed chromatin and situated singly or in clusters between apparently viable cells, in the capillaries or extracellular space.

Electron microscopy

Ultra-thin sections of 40–70 nm thickness were obtained, stained with uranyl acetate and counterstained with lead citrate and examined with a Philips CM12 transmission electron microscope.

Detection of apoptosis by labelling 3' ends of the DNA fragments with digoxigenin-11-UTP (TUNEL)

TUNEL was performed on paraformaldehyde-fixed paraffin wax-embedded tissue using the technique described by Negoescu *et al.* (1998) with modifications.

Sections (4 μm) were mounted on slides coated with BioBond (British Biocell Int, Cardiff), deparaffinized and rehydrated. Endogenous peroxide activity was blocked by immersion in 3% (v/v) H_2O_2 in methanol for 30 min. After two washes of 5 min each in 0.01 mol PBS l^{-1} (pH 7.4), slides were incubated with proteinase K (Sigma, Poole) (20 μg ml^{-1} in 20 mmol Tris l^{-1}, 2 mmol $CaCl_2$ l^{-1}, pH 7.8) for 25 min. Sections were then washed with PBS containing 5 mmol EDTA l^{-1} for 10 min at room temperature to stop the proteinase K reaction. After two 5 min washes with PBS, the slides were equilibrated with terminal deoxynucleotidyl transferase (TDT) buffer containing 30 mmol Tris–HCl l^{-1}, pH 7.2, 140 mmol sodium cacodylate l^{-1} and 1.5 mmol $CoCl_2$ l^{-1}, placed on an ice-cold plate and loaded with 2 nmol digoxigenin-11-dUTP l^{-1} and 25 U ml^{-1} TDT in enzyme buffer. Negative control slides were treated with enzyme buffer lacking TDT. The slides were immediately covered with parafilm sheets and incubated at 37°C for 75 min. The parafilm was then removed and the slides washed twice (5 min each) in standard saline citrate buffer to stop the reaction. After a further 5 min wash with PBS, sections were incubated for 10 min at room temperature with 25% (v/v) normal rabbit serum in PBS, and then incubated for 90 min at room temperature in a humidified chamber with sheep anti-digoxigenin antibody (Boehringer, Mannheim) diluted 1:100 in blocking solution. After two further washes in PBS, the slides were incubated for 30 min at room temperature with biotinylated rabbit anti-sheep immunoglobulin (Vector Laboratories, Peterborough) diluted 1:500 in PBS. Two washes in PBS (5 min each) were followed by 30 min incubation at room temperature with horseradish peroxidase–avidin biotin complex (Dako, High Wycombe) diluted in PBS according to the manufacturer's instructions. After two further washes in PBS, sections were visualized with 0.05% (w/v) 3,3'-diaminobenzidine (Sigma) in 0.05 mol Tris–HCl l^{-1}, pH 7.4, and 0.01% (v/v) H_2O_2. The sections were then washed in distilled water, lightly counterstained with haematoxylin, dehydrated in graded alcohol, cleared in xylene and mounted in DPX (distrene plasticiser xylene).

Detection of lipid droplets using oil red O stain

Frozen sections (8 μm) were stained with oil red O (Sigma) and then counterstained with haematoxylin (Filipe and Lake, 1990).

Detection of neutrophils using periodic acid–Schiff reaction

Formalin-fixed sections were stained with periodic acid–Schiff (PAS) reagent (Sigma) for 10 min and then counterstained with haematoxylin (Filipe and Lake, 1990).

Quantification method

The above stains were used on each tissue block, a minimum of three times. Two representative sections (peripheral and central) from each corpus luteum were selected and four fields per section were examined at × 400 magnification. The identity of the sections was obscured to ensure blind counting. Individual clusters of dense bodies and pyknotic nuclei that showed positive immunostaining for TUNEL, or that were identified in haematoxylin and eosin stained sections, were counted separately. The results were expressed as mean ± SEM. Sections were counted at × 400 magnification to quantify the number of neutrophils. The total number of neutrophils for each stage of the oestrous cycle was expressed as mean ± SEM. Quantimet image processing and analysis system 500 (Leica, Cambridge) was used to measure the proportion of the section stained by oil red O (area of the oil red O stain divided by the total area measured × 100). Areas were analysed at × 200 magnification. The system was optimized for each individual section on the basis of the density of the stain. The data were expressed as percentage mean ± SEM per unit area. Two sections per corpus luteum and four per section were used in the quantification, as increasing the number of sections above two sections per corpus luteum and four fields per section did not alter the result. There was no difference between the mean of the variables in central and peripheral regions, and therefore data were combined to represent one variable.

Progesterone assay

Progesterone concentrations were determined in plasma by radioimmunoassay as described by Watson *et al.* (2000). The limit of detection of the assay was 0.5 ng ml^{-1} and the intra- and interassay coefficients of variation were 9.0 and 12.6%, respectively.

Statistical analysis

The differences in early, mid-luteal, early and late regression corpora lutea were analysed by one-way analysis of variance using stage of the luteal phase as the between-subject variable. Data from untreated mid-luteal phase corpora lutea were compared with corpora lutea after induced regression using a one-way analysis of variance with treatment as the between-subject variable. The data were then subjected to Tukey's test of multicomparison among means. Results were considered to be significantly different when $P < 0.05$.

Results

Corpus luteum mass and progesterone concentrations

Morphological examination and progesterone concentrations for each corpus luteum were used to confirm the cycle stage. In three of the 23 corpora lutea, the designated stage of the cycle did not conform to the expected morphological appearance and progesterone concentration and these corpora lutea were therefore re-classified. In two corpora lutea that were collected on day 14 (early

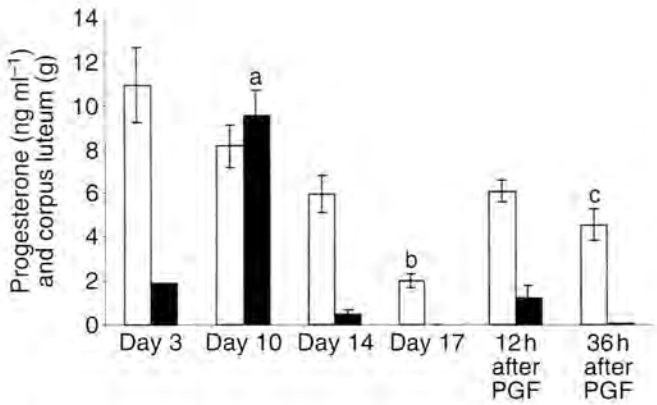

Fig. 1. Corpus luteum mass (□) and plasma progesterone concentrations (■) throughout the oestrous cycle and after $PGF_{2\alpha}$ administration (263 μg cloprostenol per 500 kg) in mares. Data are expressed as mean ± SEM. [a]Significantly ($P < 0.01$) higher than all other groups; [b]significantly ($P < 0.05$) lower than all other groups; and [c]significantly ($P < 0.05$) lower than during mid-luteal phase (day 10).

regression group), one of the animals had high progesterone concentrations (9.4 ng ml^{-1}) together with luteal cells of healthy appearance. Therefore, this animal was reclassified to the day 10 (mid-luteal phase) group. Progesterone was undetectable in the other animal and there was histological evidence of marked structural degeneration consistent with the day 17 (late regression) group. Therefore, this animal was reclassified to the late regression phase. The third corpus luteum was collected on day 17, but progesterone concentration was 1.2 ng ml^{-1} on the day of the surgery and its morphological changes coincided with those of the early luteal regression group (day 14). This animal was therefore reclassified to the early regression phase.

The mid-luteal phase was associated with maximum production of progesterone and concentrations were significantly higher ($P < 0.01$) than they were in other phases in the cycle. The time of luteolysis was day 14: progesterone concentrations on day 13 for the four corpora lutea collected in early regression were 2.1, 5.8, 2.4 and 1 ng ml^{-1}, and by day 14, progesterone concentrations had declined sharply to 0.5, 1.2, 0.5 and 0.5 ng ml^{-1}, respectively. All of the mares that were ovariectomized on day 17 had low concentrations of progesterone (< 0.5 ng ml^{-1}) for 2 days before ovariectomy. Twelve hours after $PGF_{2\alpha}$ administration on day 10, progesterone concentrations had declined significantly ($P < 0.01$) from 6.0 ± 0.01 ng ml^{-1} before $PGF_{2\alpha}$ injection to 1.0 ± 0.01 ng ml^{-1} by 12 h after the injection. By 36 h, functional regression was complete and progesterone concentrations were < 0.5 ng ml^{-1} (Fig. 1).

The corpus luteum showed changes in mass, shape and colour throughout the oestrous cycle. During the early luteal phase, the corpora lutea were irregular in shape, blood-coloured and contained a large central blood clot. By day 10, the corpus luteum became mushroom- or gourd-shaped, pink-to-purple, and four of the five corpora lutea still contained a clot of blood, although this was smaller than in the early corpus luteum. By day 14, two of four corpora lutea contained a small clot of blood, and the other two corpora lutea appeared to be homogeneous. By day 17, the corpus luteum became round, cream or yellow, and comprised a solid mass of tissue. The mean mass of luteal tissue was significantly lower ($P < 0.05$) in the late luteal phase (day 17) and 36 h after $PGF_{2\alpha}$, than in the early and mid-luteal phase (Fig. 1).

Luteal tissue histology and TUNEL immunostaining

Sections were examined by both haematoxylin and eosin and TUNEL immunostaining, and quantitative analysis of apoptosis was carried out on both sets of sections. As the morphological appearance was similar with haematoxylin and eosin and TUNEL, only the TUNEL immunostaining results are shown (Fig. 2). The luteal cells in the mid-luteal phase were polyhedral to elongated, and had abundant cytoplasm containing spherical nuclei (Fig. 2a). Pyknotic cells with densely basophilic nuclei were rarely observed. Endothelial cells had oval-to-vesicular shaped nuclei in cross-section or elongated-to-spindle shaped nuclei in longitudinal section. By day 14, luteal cells exhibited nuclear degenerative changes (Fig. 2b). Pyknotic cells with densely basophilic nuclei were observed between apparently healthy luteal cells and were classified as apoptotic cells. Discrete membrane-bound structures containing various amounts of condensed chromatin were observed singly or in clusters between apparently viable cells, in capillaries and the extracellular space, and were classified as apoptotic bodies. In late regression (day 17), all the luteal cells had decreased in size and contained vacuoles with a foamy appearance (Fig. 2c). Fibroblasts and connective tissue infiltrated the corpora lutea; intercellular debris was evident, and leucocytes were common. The nuclear changes were similar to those observed in day 14 sections. Twelve hours after $PGF_{2\alpha}$ injection, a huge influx of neutrophils was seen, and a number of cells showed pyknotic nuclei and round dense bodies were present in the extracellular space. At 36 h, most of the luteal cells were surrounded by neutrophils, and pyknotic cells and clusters of round dense bodies were abundant (Fig. 2d).

TUNEL immunostaining was found to be limited to pyknotic cells and round dense bodies. There was no immunoreactivity in interstitial cells, endothelial cells, apparently healthy luteal cells or other types of cell. Approximately 90% of pyknotic cells and dense bodies exhibited positive immunostaining with TUNEL. Positive pyknotic cells and dense bodies were seen during the mid-luteal phase (Fig. 2a), early regression (Fig. 2b), late regression (Fig. 2c), and 36 h after $PGF_{2\alpha}$ injection (Fig. 2d). Moreover, neutrophils also stained positively at 36 h after $PGF_{2\alpha}$ injection.

Quantitative analysis of haematoxylin and eosin and TUNEL-stained sections are shown (Fig. 3). Unstained

pyknotic cells and round dense bodies in TUNEL-stained sections were not included in the quantitative analysis. In early regression, the number of pyknotic cells increased ($P < 0.05$) and remained without significant change until late regression. During induced regression, there was an increase in the number of pyknotic cells in the corpora lutea collected 36 h after $PGF_{2\alpha}$ ($P < 0.05$) compared with those collected after 12 h. Clusters of round dense bodies increased in early regression ($P < 0.01$), and declined in late regression ($P < 0.01$). After induced regression there was an increase in clusters of round dense bodies 36 h after $PGF_{2\alpha}$ ($P < 0.01$) compared with 12 h after $PGF_{2\alpha}$.

Oil red O

In the early luteal phase, most of the luteal cells showed intense staining for oil red O. By the mid-luteal phase, fewer luteal cells showed positive staining (Fig. 2e). In late regression (day 17), very intense staining was observed both intracellularly and extracellularly (Fig. 2f), reflecting high accumulation of lipid in the regressing corpus luteum. Twelve hours after $PGF_{2\alpha}$ administration, few cells showed oil red O staining and by 36 h there was moderate accumulation of lipid in most of the luteal cells. The percentage area of lipid (Fig. 4) in mid-luteal phase and early regression was decreased markedly ($P < 0.01$) compared with in the early luteal phase. By late regression, the percentage area of lipid had increased sharply ($P < 0.001$).

Neutrophils

PAS stains all types of polysaccharides. Neutrophils in the equine corpus luteum stained positively with PAS (Fig. 2g). In early and mid-luteal phases (Fig. 2h), as well as in early regression, occasional neutrophils were observed, particularly in the blood vessels. On day 17, the number of neutrophils had increased slightly (Fig. 4). After $PGF_{2\alpha}$ injection at both 12 and 36 h, the number of neutrophils increased sharply ($P < 0.001$).

Ultrastructural changes

Ultrastructural examination during the early luteal phase (Fig. 5a) showed luteal cells with central or peripheral, oval-to-round shaped nuclei with one or more nucleoli. Heterochromatin was finely granular and uniformally dispersed in the nucleus. The cytoplasm contained vesicles or smooth endoplasmic reticulum (SER), electron dense bodies, ribosomes and lipid droplets. Mitochondria were round-to-elongated, and were normal in appearance with tubular and lamellar cristae. In the mid-luteal phase, luteal cells showed features similar to cells in the early luteal phase. However, the mitochondrial matrix was rarefied in some luteal cells. During regression, some luteal cells showed early stages of margination of chromatin (not shown), had fragmented chromatin (Fig. 5b), or appeared to be pyknotic (Fig. 5c). Single or clusters of dense round bodies (Fig. 5d) were observed in the extracellular space or in the capillaries (Fig. 5f). Other luteal cells showed non-apoptotic changes with shrinkage, crenation and involution of the nuclear membrane (Fig. 5e). The luteal cells showed accumulation of lipid, and disruption in both SER and mitochondria (Fig. 5b,c,e).

Endothelial cells were seen in the capillaries with oval-to-vesicular shaped nuclei, and contained aggregated dispersed heterochromatin with complex infoldings of the nuclear envelope during the mid-luteal phase (Fig. 6a). During regression, the endothelial cells appeared to protrude into the blood vessel lumens and were swollen or detached from the plasma membrane (Figs 5d and 6b). Other cell types, such as macrophages, were abundant, engulfing apoptotic bodies or degenerated luteal cells (Fig. 6c). A marked influx of neutrophils was seen only during induced regression (Fig. 6d).

Discussion

This report has described cell death during natural and induced luteal regression in mares, combining, for the first time, morphological changes observed under light and electron microscopy with immunostaining for the 3′ end labelling of DNA (TUNEL technique). This is also the first description, to the author's knowledge, of cellular changes during $PGF_{2\alpha}$-induced luteolysis in mares. The detection of pyknotic luteal cells and round dense bodies that stained positively with TUNEL during natural and $PGF_{2\alpha}$-induced regression was strongly indicative of apoptosis. However, the crenation of the nuclear membrane and the shrinkage of the nuclei in the luteal cells were indicative of an additional form of cell death, possibly terminal differentiation.

In the present study, rarefied mitochondria indicative of minor degenerative changes were observed in some luteal cells in the mid-luteal phase (day 10), which is around the time of onset of declining progesterone concentrations in the oestrous cycle in mares (Van Niekerk *et al.*, 1975). By day 14, and by 12 h after $PGF_{2\alpha}$ administration on day 10, marked structural degenerative changes were detected and circulating progesterone concentration had fallen to < 1 ng ml^{-1}. Overall, these results indicate that marked structural changes did not precede the decrease in progesterone concentrations. These results are in agreement with studies in ruminants (Umo, 1975; Juengel *et al.*, 1993; Zheng *et al.*, 1994) and monkeys (Young *et al.*, 1997).

The degeneration of the mitochondria in the mid-luteal phase in mares is an early step in the decline in progesterone concentrations, since the mitochondria are involved in steroidogenesis (Levine *et al.*, 1979). Recent studies in other tissues indicate that disruption of mitochondria reflects early signs of apoptosis (Kroemer *et al.*, 1998; Wilson, 1998). Since apoptotic cell death is regulated by oncoproteins in the mitochondria (Brenner *et al.*, 1998), this period of regression may be the key time for further study of changes in the expression of the oncoproteins that initiate the cell death process.

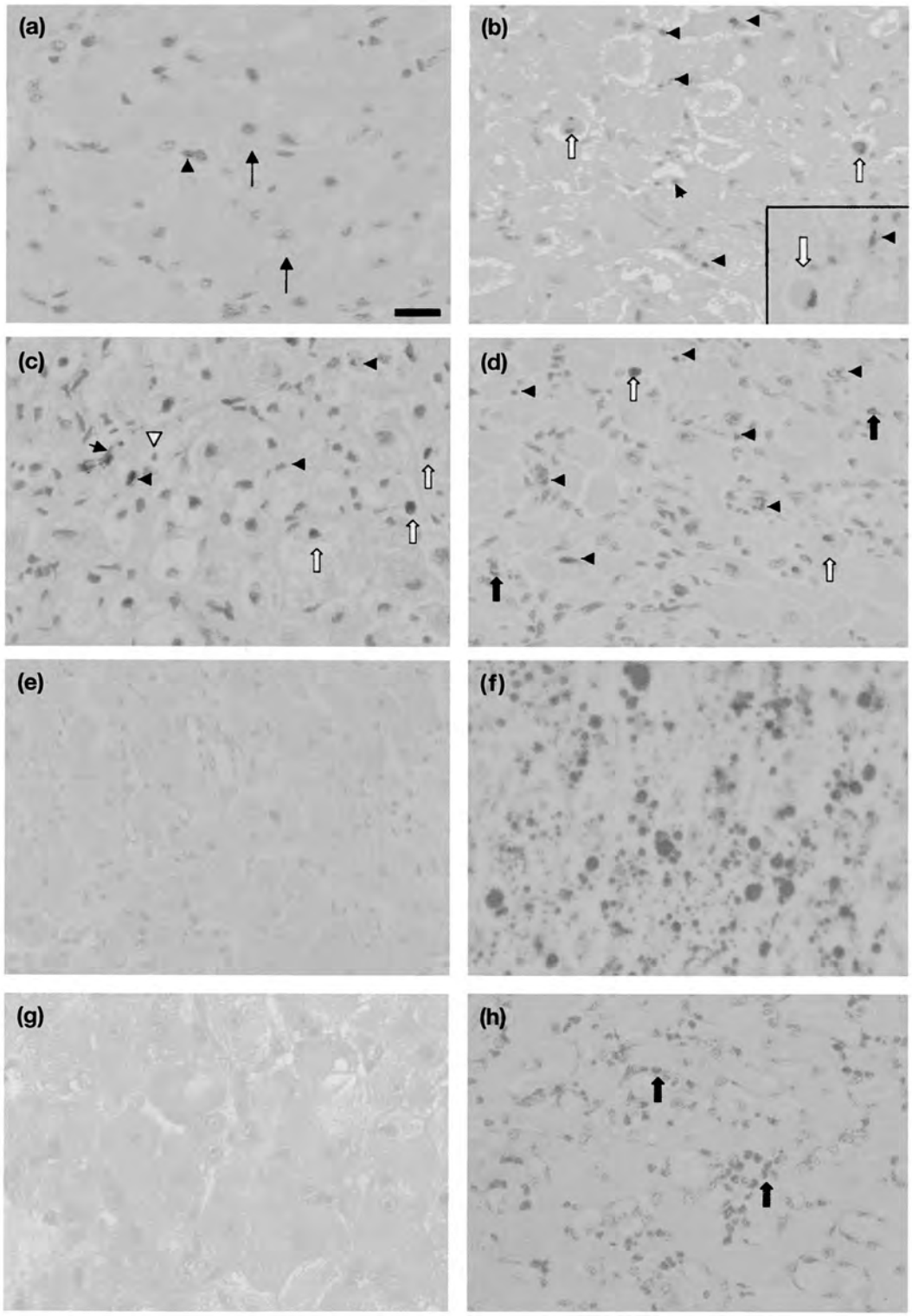

Fig. 2. TUNEL immunostaining in paraformaldehyde-fixed tissue; oil red O staining in frozen sections and periodic acid–Schiff (PAS) staining in formalin fixed tissue from the corpora lutea of mares. (a) Mid-luteal phase corpus luteum (day 10) showing luteal cells with healthy nuclei. One apoptotic round dense body is present. (b) Positive immunostaining of pyknotic cells and round dense bodies in the corpus luteum in early regression (day 14). Inset shows high magnification of apoptotic cell and round dense bodies. (c) Immunopositive staining in late regression corpus luteum (day 17) for both pyknotic cells and round dense bodies. (d) Corpus luteum 36 h after $PGF_{2\alpha}$ administration, neutrophils, pyknotic cells and round

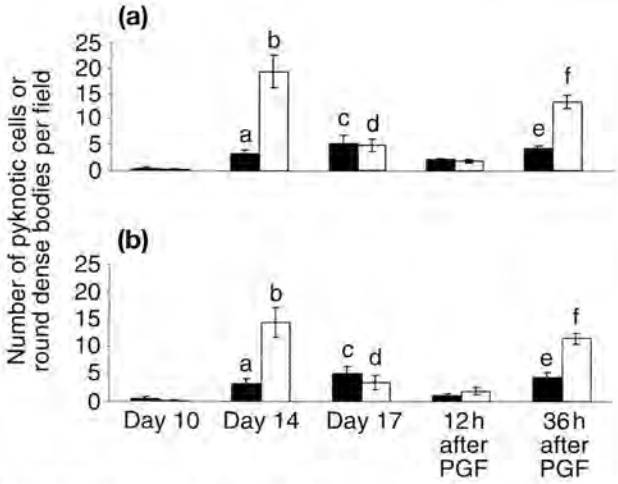

Fig. 3. Number of pyknotic cells (■) and round dense bodies (□) per field at × 400 magnification expressed as mean ± SEM in equine luteal tissue (n = 3–4 animals for each phase) during induced and natural regression. (a) TUNEL immunostaining; (b) paraformaldehyde-fixed tissue stained with haematoxylin and eosin. [a]Significantly ($P < 0.05$) higher than during mid-luteal phase (day 10); [b]significantly ($P < 0.01$) higher than during late regression phase (day 17); [c,d]significantly ($P < 0.05$) higher than during mid-luteal phase (day 10); [e]significantly ($P < 0.05$) higher than during mid-luteal phase (day 10) and 12 h after $PGF_{2\alpha}$ administration; [f]significantly ($P < 0.01$) higher than during mid-luteal phase (day 10) and 12 h after $PGF_{2\alpha}$ administration.

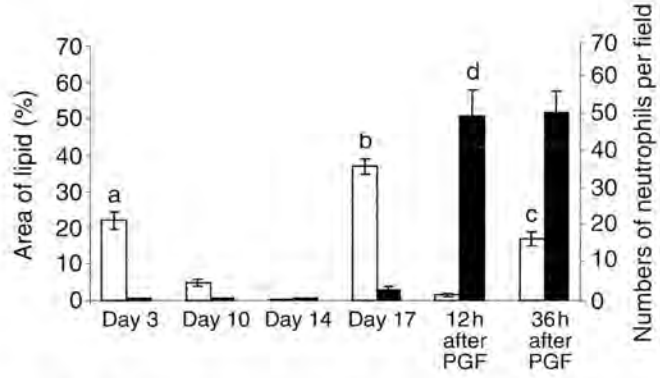

Fig. 4. The area fraction for oil red O stain (□) and number of neutrophils (■) in equine luteal tissue, expressed as mean ± SEM, during natural and induced regression. [a]Significantly ($P < 0.01$) higher than during mid-luteal phase (day 10); [b]significantly ($P < 0.001$) higher than during mid-luteal (day 10) and early regression phases (day 14); [c]significantly ($P < 0.01$) higher than during mid-luteal phase (day 10) and 12 h after $PGF_{2\alpha}$ administration; [d]significantly ($P < 0.001$) higher than during mid-luteal phase (day 10).

In the present study, both TUNEL technique and morphological examination under light and electron microscopy demonstrated the presence of pyknotic cells and round dense clusters of chromatin in the extracellular space, characteristic of apoptosis. The pathways of cell degeneration in natural and induced regression appeared to be broadly similar. It appears that luteal regression in mares may involve apoptosis in the elimination of degenerated and unwanted cells from the corpus luteum, in agreement with a previous ultrastructural study in sheep that showed apoptosis in luteal cells (Sawyer *et al.*, 1991).

In the present study, TUNEL immunostaining was limited to pyknotic cells and round dense bodies. Paraffin wax-embedded histological sections of marmoset corpus luteum have been shown to exhibit apoptosis both morphologically and immunohistochemically (by TUNEL) (Young *et al.*, 1997). However, resin sections from the same tissue did not show the ultrastructural features associated with classic apoptosis, but rather autophagocytosis and nonlysosmal disintegration, and it may be that TUNEL over-represented apoptotic degeneration by staining non-apoptotic chromatin (Fraser *et al.*, 1999). Although many studies have used TUNEL to detect apoptosis, few have investigated the death of the luteal cells ultrastructurally. In the present study, results from haematoxylin and eosin staining and TUNEL were confirmed by electron microscopy, highlighting the importance of ultrastructural examination of tissues in investigating cell death during regression.

When haematoxylin and eosin and TUNEL staining were used to quantitate changes in the corpus luteum during regression, the peak increase in round dense bodies occurred in the early luteal phase (day 14), and was followed by the sharp decrease during late natural regression (day 17), indicating an active removal mechanism. It is not clear whether ultimately these round dense bodies undergo phagocytosis or are flushed out of the corpus luteum via the blood supply, but data from the present and previous studies indicate that elimination by macrophages is likely (Wyllie *et al.*, 1980; Manjog and Joris, 1995). The increased numbers of macrophages during luteal regression, observed ultrastructurally in the present study and immunocytochemically in that of Lawler *et al.* (1999), indicate a role for macrophages in the removal of apoptotic bodies.

The crenation of the nuclear membrane with shrinkage of the nucleus seen in some luteal cells during luteal

dense bodies show positive immunostaining. (e) Oil red O staining in mid-luteal phase corpus luteum showing lipid droplets in a few luteal cells. (f) Accumulation of lipid droplets in late regression corpus luteum. (g) Mid-luteal phase corpus luteum stained with PAS showing healthy normal cells and no neutrophils. (h) Corpus luteum 12 h after $PGF_{2\alpha}$-induced luteolysis, showing a huge influx of neutrophils associated with vasodilation. Thin black arrows indicate healthy luteal cells, thick white and black arrows indicate pyknotic cells and neutrophils, respectively, black and white arrowheads indicate round dense bodies and unstained round dense bodies, respectively. Scale bar represents 20 μm and 10 μm (inset).

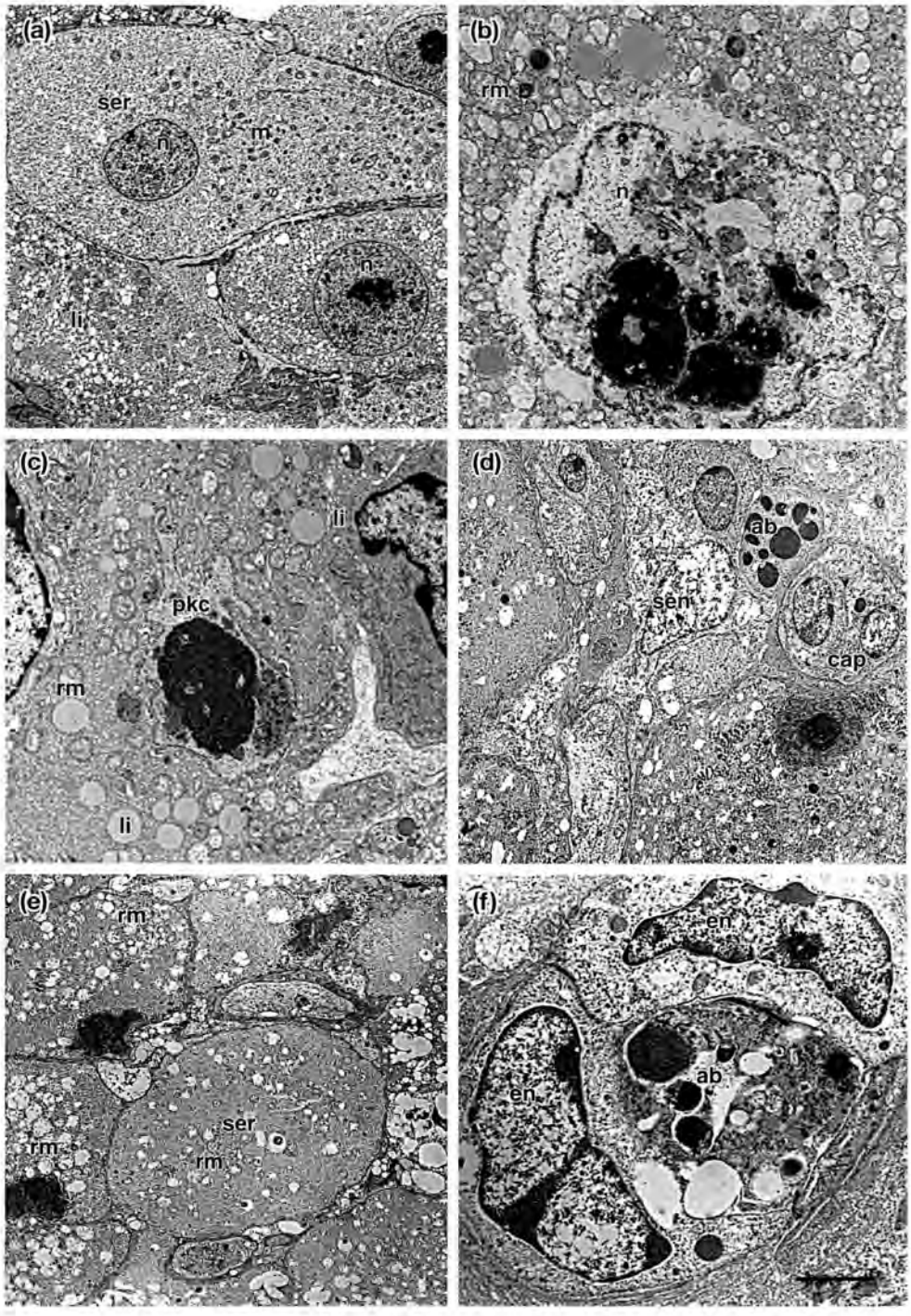

Fig. 5. Ultrastructural changes in the mare corpus luteum during natural regression. (a) Luteal cells in early luteal phase (day 3) showing typical structure of hormone producing cells, with spherical nuclei (n), healthy mitochondria (m) and smooth endoplasmic reticulum (ser) and lipid droplets (li). (b) Degenerated luteal cell in early regression phase showing nucleus (n) with fragmented masses of chromatin, shrinkage in the nuclear membrane and rarefied mitochondria (rm). (c) Luteal cell in late regression phase (day 17) showing chromatin with pyknotic appearance (pkc), lipid droplets and rarefied mitochondria. (d) Early regression phase (day 14) corpus luteum showing cluster of round dense bodies, believed to be apoptotic bodies (ab) in the extracellular space, a capillary (cap) with two swollen endothelial cells, and another endothelial cell with swelling (sen). (e) Luteal cells in early regression showing crenation of the nuclear membrane and shrinkage of the nucleus, condensation of the smooth endoplasmic reticulum and rarefied mitochondria (rm). (f) Late regression (day 17) showing capillary with endothelial cells (en) obstructed by apoptotic bodies and debris. Scale bar represents (a) 3, (b) 0.1, (c) 1, (d) 1.5, (e) 2 and (f) 0.3 μm.

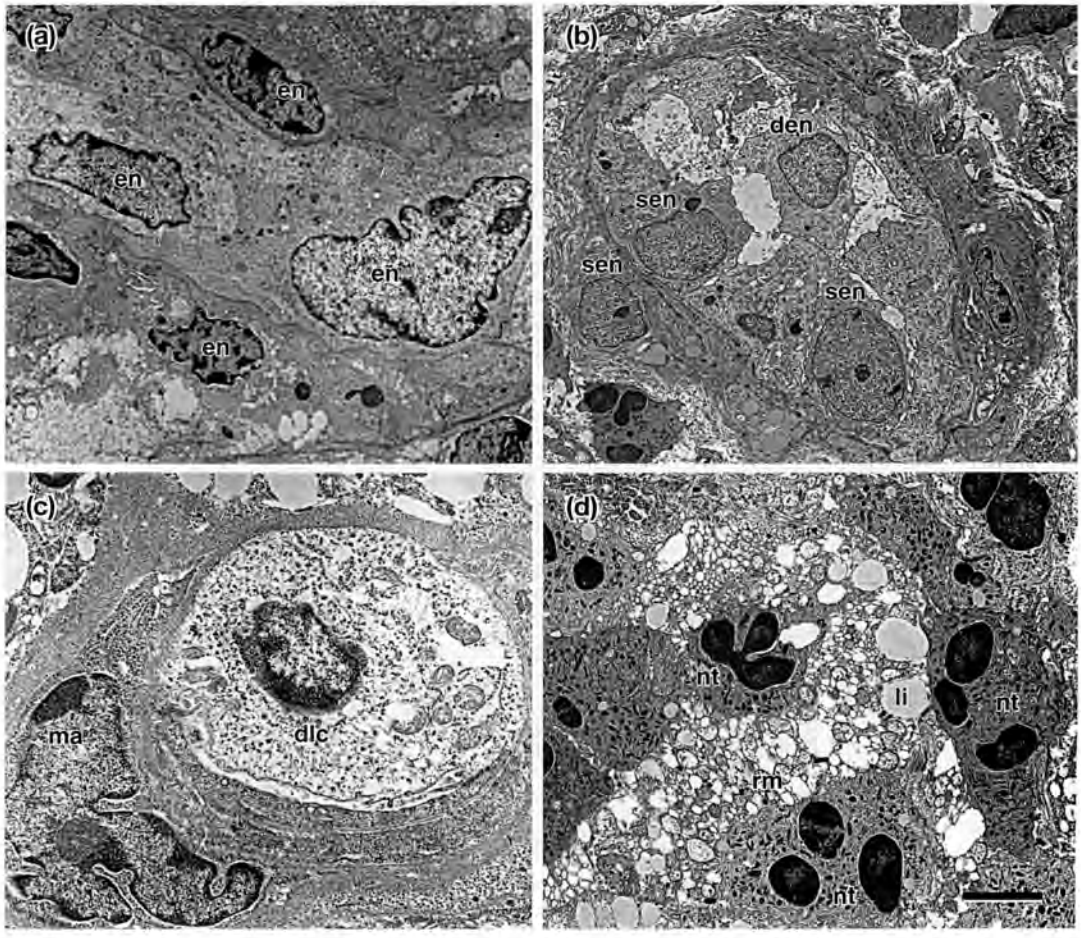

Fig. 6. Ultrastructural changes in the equine corpus luteum throughout late regression and after $PGF_{2\alpha}$-induced luteolysis. (a) Healthy endothelial cells (en) in mid-luteal phase showing elongated flattened shape with jagged membrane and a dense mass of chromatin in the nucleus. (b) Endothelial cells appeared to protrude into the blood vessel lumens swollen (sen) and detached from the plasma membrane (den) during induced regression. (c) Macrophage (ma) engulfing whole degenerated luteal cell (dlc) during late regression phase. (d) Massive influx of neutrophils (nt), lipid droplets (li) and rarified mitochondria (rm) 36 h after $PGF_{2\alpha}$ administration. Scale bar represents (a,d) 2, (b) 3 and (c) 1 μm.

regression in mares may indicate the presence of an additional non-apoptotic form of cell death at luteolysis. It is possible that these cells were undergoing the type of cell death known as terminal differentiation in which progressive condensation of nuclear material results eventually in pyknosis and nuclear destruction or expulsion (van Wezel *et al.*, 1999). In the regressing corpus luteum of marmosets, autophagocytosis and nonlysosmal disintegration may occur (Fraser *et al.*, 1999). In autophagocytosis, the nucleus disintegrates and is digested by autolysosomes and, in nonlysosmal disintegration, organelles swell and formation of vacuoles and cytoplasmic destruction is followed by nucleus disintegration. However, these changes were not observed in the present study in mares.

TUNEL and ultrastructural examination confirmed that endothelial cells did not show any morphological signs of apoptosis, but that they did show signs of swelling and detachment from the walls of the blood vessels. Ultrastructural studies have shown that apoptosis occurs in endothelial cells during regression of the corpora lutea in guinea-pigs (Azmi and O'Shea, 1984) and in sheep after $PGF_{2\alpha}$-induced regression (Sawyer *et al.*, 1990), but that it does not occur in cows (Augustin *et al.*, 1995; Modlich *et al.*, 1996). These studies strongly indicate that there are species differences in the fate of endothelial cells during luteolysis, and that the vasculature in bovine and equine corpora lutea regresses in a similar manner.

This is the first report, to the authors' knowledge, showing infiltration of neutrophils into the corpus luteum after $PGF_{2\alpha}$-induced luteolysis in domestic animals. An intense infiltration of neutrophils was observed 12 and 36 h after $PGF_{2\alpha}$ administration. Influx of neutrophils during

luteal regression has been reported during spontaneous regression in hamsters, indicating that neutrophils assist in initiating luteal regression in this species (McCormack *et al.*, 1998). Although the essential function of neutrophils is phagocytosis, they also initiate and modify the magnitude and duration of the acute inflammatory processes (Jain, 1993). It has been proposed by studies in the hamster corpus luteum that these cells play a role in luteal regression via cytokine secretion (McCormack *et al.*, 1998).

A marked increase in lipid droplets during luteal regression was observed. This accumulation of lipid was seen in late regression (day 17), but not in early regression (day 14), indicating that accumulation of lipid is a feature of late regression, occurring after the onset of structural regression. This time course of events was confirmed by observations after $PGF_{2\alpha}$-induced luteolysis. At 12 h after $PGF_{2\alpha}$ administration, there was no lipid accumulation, despite the onset of structural regression, but by 36 h after $PGF_{2\alpha}$ administration, there was a marked lipid accumulation. Accumulation of lipid is associated with corpus luteum regression in sheep (Deane *et al.*, 1966; Umo, 1975), cows (Priedkalns and Weber, 1968), sows (Waterman, 1980), rats (Guraya, 1975) and marmosets (Young *et al.*, 1997). The exact mechanism by which lipid accumulation occurs during regression is not well understood. A degeneration in mitochondria and SER has been proposed to result in a decline in progesterone production and to play a role in the accumulation of lipid (Umo, 1975; Levine *et al.*, 1979). However, the present study showed that accumulation of lipid does not occur directly in response to a decline in progesterone production and the luteolytic effect of $PGF_{2\alpha}$, but requires a period of time to develop.

In conclusion, degenerative changes in the equine corpus luteum may be initiated by day 10 (mitochondrial rarefaction), and apoptotic bodies and cells were present by day 14 of the cycle and 12 h after $PGF_{2\alpha}$ injection. Non-apoptotic changes were also observed in some luteal cells during regression. Lipid accumulation is a feature of late regression. A marked influx of neutrophils was observed after $PGF_{2\alpha}$-induced regression.

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CHANGES IN THE VASCULATURE OF THE EQUINE CORPUS LUTEUM THROUGHOUT THE OESTROUS CYCLE AND AFTER $PGF_{2\alpha}$ INDUCED LUTEOLYSIS

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INTRODUCTION: Changes in microvessels have been suggested to play an essential role in CL growth and regression. Understanding the process of luteal angiogenesis in the mare could provide a more precise control over the oestrous cycle. The current study evaluated the vascular changes in the equine CL throughout the oestrous cycle and after $PGF_{2\alpha}$ administration.

MATERIALS AND METHODS: CLs were obtained during early (Day 4, n=4), mid (Day 10, n=4), early regression (Day 14, n=4), late regression (Day 17, n=4), and 12 and 36h (n=3 each) following $PGF_{2\alpha}$ administration on day 10 and fixed in Formalin. Blood vessels were identified by immunohistochemical staining of Factor VIII. An estimation of blood vessel volume density in both trabeculae and luteal tissue, as well as the percentage of vessels with lumen, was determined by morphometry.

RESULTS AND DISCUSSION: After ovulation, sprouting endothelial cells invaded the cavity of the CL and continued to grow until the mid luteal phase. The mid luteal CL was characterised by a dense network of fully differentiated capillaries with elongated flattened endothelial cells; vascularization was increased significantly ($P< 0.05$) compared with the early luteal CL. During late natural luteolysis, there was massive degeneration and a decline of approximately 50% in the volume density of luteal tissue, accompanied by a significant decrease in capillary density ($P< 0.05$). Interestingly, 12 h after $PGF_{2\alpha}$ injection, influx of neutrophils and vasodilation associated with an increase in vascularity was observed ($P< 0.001$). These changes were short lived, and by 36 h there was a decline in the volume density of the microvessels without a decline in neutrophil numbers. These findings suggest a temporal pattern of blood vessel growth and regression during the ovarian cycle. To our knowledge, this is the first report showing an increase in blood vessels and vasodilation 12 h after $PGF_{2\alpha}$ administration which may reflect the high sensitivity of the mare to $PGF_{2\alpha}$.

ELSEVIER

EFFECT OF INSEMINATION TIME OF FROZEN SEMEN ON INCIDENCE OF UTERINE FLUID IN MARES

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ABSTRACT

Ninety five mares were inseminated with frozen semen either within 12 h before ovulation or within 8 h after ovulation. The effect of preovulatory versus postovulatory insemination (AI) on the subsequent detection of uterine fluid was studied. The overall pregnancy rate was 43% and this was not significantly influenced by preovulatory or postovulatory insemination. When mares were first examined 12 h after AI, 18 of 52 mares (35%) had accumulated uterine fluid. However, when mares were first examined 18 to 24 h after AI, only 6 of 43 mares (14%) had uterine fluid. Presence of intrauterine fluid significantly lowered pregnancy rates. Timing of insemination did not affect incidence of uterine fluid. Serum concentrations of estrogen and progesterone at time of insemination did not influence uterine clearance or pregnancy rates, but both hormones were higher at preovulatory than at postovulatory inseminations. We concluded that there was no evidence that postovulatory inseminations would predispose mares to persistence of uterine fluid after AI.

Key words: mare, insemination, endometritis

INTRODUCTION

Use of frozen semen for insemination of mares is increasing significantly throughout the world. Improvement in freezing techniques has resulted in pregnancy rates of greater than 30% per cycle (3, 14, 19, 25). These rates change depending on the number of post-thaw motile spermatozoa per inseminate, on the proximity of insemination to time of ovulation and the number of inseminations per estrus (10, 19, 25, 26).

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The use of a small volume of highly concentrated spermatozoa in uterine insemination in mares has resulted in a more marked inflammatory response than did the use of a larger volume of inseminate (9, 15). It has been shown in vitro that equine spermatozoa are chemotactic for neutrophils (23). Addition of seminal plasma was shown to suppress the chemotactic effect of the spermatozoa in vitro (23), but addition of seminal plasma to sperm did not suppress the influx of neutrophils into the uterus of the mare in vivo (9). The low volume used in insemination of frozen semen may be an important factor (9, 15). With large insemination volumes, considerable amounts of fluid and spermatozoa are discharged into the vagina shortly after insemination (20), whereas with low volumes, it is likely that the spermatozoa are in contact with the endometrium for a longer period, causing neutrophil influx into the lumen by their chemotactic properties. Although many studies have reported the effect of different freezing procedures on pregnancy rates in mares, few field studies have investigated the uterine response to AI with frozen semen and its effect on pregnancy rates. An inflammatory response in the mare's uterus in response to insemination may be monitored ultrasonographically by the presence of fluid accumulations within the uterine lumen (22). As increased circulating concentrations of progesterone are associated with suppression of both uterine immune defense mechanisms and uterine clearance (4, 29, 30), and as progesterone increases within hours of ovulation in the mare (21), we hypothesized that insemination in the postovulatory period might predispose mares to post-insemination fluid accumulation, compared with preovulatory insemination.

We report the outcome of inseminations performed at a center in Scotland in 1999 and in Italy in 1998 with respect to the presence of ultrasonographically detectable intrauterine fluid after insemination, whether this was influenced by timing of insemination, or by circulating steroid hormone concentrations at the time of insemination.

MATERIALS AND METHODS

Records were available from a total of 95 mares at the R(D)SVS, Scotland and the Cristella Veterinary Clinic in Italy. Only first inseminations were included. The mares were aged 3 to 16 years and most were Warmbloods, with a few quarterhorses and Thoroughbreds. Uterine swabs, collected from all mares early in the same estrus as AI (5), were negative on bacteriological culture and cytological examination. None of the mares had more than a trace of uterine fluid at the time of insemination. Semen from 42 stallions frozen at 18 different centers was used. The semen was packaged mainly in 0.5 mL straws, however 5 mL macrotubes were used at one center. The minimum number of post-thaw progressively motile spermatozoa inseminated was 100 million.

Timing of Insemination

If mares were in late diestrus or early estrus, they were scanned daily by transrectal ultrasonography until a follicle of at least 35 mm in diameter was present in the ovaries, along with a positive tease response to a stallion and ultrasonographically visible uterine edema. These

mares were treated with human chorionic gonadotrophin intravenously (hCG; 2000IU[1]) on first day of detection of a follicle of 30 to 40 mm in diameter. In some of the cycles, mares were not presented until later in estrus when large follicles >40 mm were present. These mares either received hCG or were scanned every 4 to 6 h until ovulation depending on the size and degree of softening of the follicle. The ovaries and uterus of mares were examined by transrectal ultrasonography at 12 and 24 h after hCG and then every 4 to 8 h until ovulation. Jugular blood samples were collected at the time of insemination from 52 of the mares into plain evacuated tubes. Serum was separated and stored at -20°C until assayed for estrogen and progesterone.

Mares were inseminated once only, either within 4 to 8 h after ovulation was detected, or around 36 h after hCG treatment if the mare had not yet ovulated (n = 32). All of these 32 mares ovulated within 12 h after insemination. Mares were prepared for insemination as described previously (27) and semen was thawed according to the semen distribution center's instructions. The semen was inseminated immediately and then a drop was checked for progressive motility in the laboratory. All semen inseminated was at least 30% progressively motile after thawing.

Post-Insemination Monitoring

Transrectal ultrasonography was performed either 12 (n = 52) or 18 to 24 h (n = 43) after insemination. The presence and depth of intrauterine fluid was recorded. Fifteen mm of intrauterine fluid was recorded as a significant volume as this amount of fluid in diestrus reduces pregnancy rates (13). Mares with less than 15 mm intrauterine fluid received an intravenous injection of oxytocin (20 IU; Oxytocin S[2]). In mares with greater than 15 mm intrauterine fluid, buffered saline solution was infused and recovered (one liter aliquots) until the recovered fluid was clear. The mares then received oxytocin (20 IU iv). Oxytocin treatment was repeated up to three times on that day. The combination of lavage and oxytocin was repeated daily as necessary until no intrauterine fluid was detected on ultrasound scan, for up to three days after insemination. The mares were scanned for pregnancy between 14 and 17 days postovulation.

Hormone Assays

Progesterone and estradiol-17β were measured by a solid phase, chemiluminescent enzyme immunoassay at Beaufort Cottage Laboratories, Newmarket. The assays were performed as described by Reimers et al. (18) and Ousey et al. (16). Major cross-reactivities of the progesterone antibody were with progesterone (100%), 5α-pregnane-3,20-dione (1.6%), 17α-hydroxyprogesterone (1%), 11–deoxycorticosterone (1.2%) and of the estradiol antibody were with estradiol-17β (100%), α-equilenin (1.6%) and ethinyl estradiol (1.2%). Sensitivity of the progesterone assay was 0.2 ng/mL and of the estradiol assay was 12 pg/mL. Mean intra- and inter-assay coefficients of variation for progesterone for a range of concentrations were 8.3% and 9.2%, respectively, and for estradiol were 11% and 12.4%, respectively.

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Statistical Analysis

Effect of age, intrauterine fluid, and timing of ovulation on pregnancy rates and effects of hCG were analyzed by a Chi-square test. Hormone concentrations were compared using a student's t test. Differences of $P < 0.05$ were regarded as significant.

RESULTS

Forty-one of the 95 inseminations resulted in a pregnancy (43%). None of the mares included was older than 16 years, and age (3 to 9 years, n = 55 versus 10 to 16 years, n = 40) did not influence pregnancy rate (43% for younger mares v 43% for older mares). Timing of insemination (within 12 h before ovulation, compared with within 8 h after ovulation) did not significantly affect pregnancy rates (13/32, 41% with preovulation insemination versus 28/63, 44% with postovulation insemination). Similarly, timing of insemination did not significantly influence accumulation of intrauterine fluid 12 h later (34% of mares accumulated fluid after preovulatory insemination compared with 21% after postovulatory insemination).

Transrectal ultrasonography was performed 12 h after 52 of the inseminations and 18 to 24 h after 43 of the inseminations (Table 1). Of the mares examined 12 h later, 18 (35%) had ≥15 mm intrauterine fluid. Pregnancy rates in these 18 mares were significantly lower ($P < 0.05$) in mares with fluid compared to mares with no fluid or only a small amount. Of the 18 mares with fluid at 12 h, 10 were in the older age group (56%, 10 to 16 years). This difference was not significant. Only 6 of the mares examined for the first time after 18 to 24 h had ≥15 mm intrauterine fluid although the difference in detection rates between scanning early (12 h) versus later (18 to 24 h) failed to reach significance ($P = 0.07$). Of these 6 mares, three became pregnant (50%) compared with 15 (41%) of the mares with little or no fluid.

Table 1. Presence of intrauterine fluid (IUF) at 12 and 18 to 24 h after insemination and effects on pregnancy rates

	First scanned	
	12 h post AI	18 to 24 h post AI
presence of IUF	18/52 (35%)	6/43 (14%)
pregnancy rates in mares with IUF	5/18 (28%)*	3/6 (50%)
pregnancy rates in mares without IUF	18/34 (53%)*	15/37 (41%)

*$P < 0.05$

Concentrations of progesterone and estrogen at time of insemination did not significantly affect pregnancy rates and progesterone concentrations were not significantly higher at the time of insemination in those mares that were inseminated postovulation (Table 2). However, serum

estrogen and progesterone concentrations were significantly higher at the time of preovulatory insemination.

Concentrations of circulating estrogen and progesterone at insemination were not related to presence of intrauterine fluid 12 h later (Table 2). Number of large follicles (≥ 35mm) at time of ovulation did not influence circulating concentrations of estrogen or progesterone (Table 2).

Table 2. Serum concentrations of estrogen and progesterone at AI

		Hormone concentrations at time of AI	
	n	Estrogen pg/mL	Progesterone ng/mL
Pregnant	23	36.6 ± 6.35	0.21 ± 0.03
Not pregnant	29	43.2 ± 8.93	0.28 ± 0.04
Preovulatory AI	32	48.9 ± 8.73 *	0.28 ± 0.03 *
Postovulatory AI	20	26.3 ± 2.72 *	0.15 ± 0.03 *
≥ 15mm uterine fluid } 12 h post AI	18	39.5 ± 10.99	0.28 ± 0.06
< 15mm uterine fluid } 12 h post AI	34	40.3 ± 6.24	0.22 ± 0.03
Single follicle } at AI or at last scan	25	38.9 ± 7.08	0.24 ± 0.03
Two follicles ≥ 35mm } at AI or at last scan	27	40.3 ± 8.72	0.25 ± 0.03

* Values significantly different ($P < 0.01$) within columns.

DISCUSSION

We showed that timing of insemination, either before or after ovulation, did not influence accumulation of uterine fluid 12 h later. Similarly circulating concentrations of steroid hormones at time of insemination were not related to subsequent accumulation of uterine fluid. In the present study, pregnancy rates were similar to those previously reported for insemination of frozen semen (2, 3, 7, 10, 25, 27). The stallion, dose and quality of spermatozoa and number of inseminations all have a profound effect on single cycle pregnancy rates (10, 19, 25). However, in agreement with recent studies, in our mares timing of insemination within a period of 12 h before, or 4 to 8 h after ovulation did not significantly affect pregnancy rates (2, 3). By contrast, Samper (19) stated that a single preovulatory insemination resulted in higher pregnancy rates (60%) than insemination after ovulation (30%). However, this author was referring to within a period of 12 h postovulation and it has been shown that pregnancy rates fall as the oocyte ages between 6 and 12 h (11). The high pregnancy rate quoted by Samper (19) for a single

preovulatory insemination was also achieved by postovulatory insemination for individual stallions in the present study, but not by other stallions. Other workers quoted similarly high rates for postovulation insemination in research situations using a small number of stallions (8, 12). This high rate is unlikely to be obtained in practice when a large number of commercial stallions are used.

More mares first examined by transrectal ultrasonography at 12 h after insemination had intrauterine fluid (35%) than those first examined between 18 and 24 h after AI (14%), although this difference failed to reach statistical significance. The higher incidence 12 h after AI is not surprising as intrauterine inflammation peaks eight hours after AI with fresh semen (6). Time of first detection of fluid after AI did not significantly influence pregnancy rates, and pregnancy rates were not reduced when treatment was not instituted until 18 to 24 h after AI. Indeed it seems likely that a number of mares were treated unnecessarily after the earlier scan. More work needs to be performed on the effect of timing of first treatment on subsequent pregnancy rates in mares with delayed uterine clearance.

However, the overall low pregnancy rate in mares with intrauterine fluid (33%) does suggest that these mares need treatment. Other studies showed that mares with intraluminal fluid within 48 h of mating have lower pregnancy rates than do mares with no intraluminal fluid (20, 31) and mares with fluid at any time in diestrus after insemination with fresh semen have profoundly reduced pregnancy rates (1). Treatment of mares that retain intraluminal fluid has been shown to aid fluid removal and increase pregnancy rates (17, 24). The current recommendations for treatment regimens are as performed in the present study, that is oxytocin therapy with or without large volume intrauterine lavage, depending on the volume of fluid retained (22). Unfortunately, in the present study we could not leave mares with intrauterine fluid untreated to study the effect of treatment on pregnancy rates, but the low pregnancy rates achieved in these mares and mares in other studies would suggest that an optimal treatment regimen remains to be devised.

In the present study the incidence of moderate to large accumulations of fluid on the day after insemination with frozen semen was similar to that reported after natural mating (16% of cycles) (31). This is surprising in that intrauterine infusion of frozen semen has been shown to result in a significantly greater inflammatory response than natural breeding (9). These latter authors hypothesized that the intensity of neutrophil reaction depended on the concentration and/or volume of inseminate. More recently Troedsson and co-workers (23) showed that seminal plasma suppressed neutrophil migration in vitro and then Nikolakopoulos & Watson (15) showed that larger infusion volumes were associated with a reduced neutrophil response in mares. However in the present study the population of mares was relatively young, and mares with a history of low fertility were not accepted as candidates for insemination with frozen semen. If an unselected population had been used, it is likely that fluid accumulation rates would have been higher. Indeed it has been shown that fluid accumulation after insemination of frozen semen is higher in mares over 16 years old (3).

Concentrations of estrogen and progesterone were significantly higher at the time of insemination when insemination was carried out in the preovulatory period. The high estrogen at this time would derive from the preovulatory follicle, the main source of estradiol 17-β in the mare. The higher levels of circulating progesterone were unexpected. However it has been shown previously that intrafollicular concentrations of progesterone increase after hCG treatment (28) and so it is possible that the preovulatory follicle secretes more progesterone than the newly ovulated corpus hemorrhagicum. Pregnancy rates were not affected by circulating steroid concentrations at the time of AI. Circulating steroid concentrations at AI did not influence accumulation of intrauterine fluid after insemination and so we had no evidence that postovulatory insemination could predispose to persistent mating-induced endometritis. Indeed in our study progesterone concentrations were higher before ovulation. There was no evidence that mares with two follicles had higher circulating estrogen concentrations than mares with one large follicle. Therefore increased numbers of follicles did not appear to confer mares with higher estrogen levels that might be beneficial in uterine defense and clearance.

In conclusion, accumulation of intrauterine fluid in mares after insemination with frozen semen was not significantly affected by timing of insemination before or after ovulation. Furthermore there was no correlation between circulating concentrations of estrogen and progesterone at time of insemination and post-insemination fluid accumulation. Fewer mares had intrauterine fluid on the day after AI than at 12 h after AI, and so routine treatment as early as 12 h will include some normal fertile mares.

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Abstract

Can uterine contractile activity be evaluated by transrectal ultrasonography?

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1. Introduction

Uterine contractile activity (UCA) has been studied in many species, including the mare, employing different techniques such as intrauterine pressure catheters, scintigraphy, electromyography and ultrasonography. Transrectal ultrasonography remains a technique not widely used for the clinical evaluation of UCA in the mare. The aim of the present study was to assess the use of transrectal ultrasonography in the measurement of UCA and to monitor the effect of oxytocin (OT) on UCA in estrous and diestrous mares and in mares susceptible to persistent mating-induced endometritis (PMIE).

2. Materials and methods

A group of mares resistant ($n = 12$, 5–15 years, 370–520 kg) and susceptible ($n = 10$, 7–18 years, 370–620 kg) to PMIE were used. We investigated the effect of transrectal ultrasonography on UCA, and on circulating concentrations of OT and PGFM in resistant estrous mares ($n = 5$) by recording UCA on videotape for 10 min and measuring OT and PGFM in blood samples collected at 1 min intervals during the recording. The effect of OT administration on UCA in resistant and susceptible estrous and diestrous (D7) mares was recorded on video for 3 min before and then for 10 min after i.v. administration of OT (1 IU/20 kg). Concentrations of OT and PGFM were measured by RIA as described previously [1,2]. UCA was assessed and scored from 1 min segments of videotape. UCA represents both synchronous and uncoordinated uterine activity. UCA was scored between 1 (minimum) and 10 (maximum). The linear relationship between mean UCA scores and mean OT and PGFM concentrations was tested using regression analysis. Mean UCA scores of estrous and diestrous resistant and susceptible mares were compared using a two

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sample t-test. Mean baseline UCA scores prior to OT administration were compared to mean scores of the control groups using a paired t-test and the duration of the OT effect on UCA between resistant and susceptible estrous mares and the differences between mean baseline PGFM concentrations were compared using a two sample t-test.

3. Results

Mean OT and PGFM levels, and mean UCA scores remained constant and did not change significantly at any time throughout the 10 min scanning procedure in the five estrous mares (data not shown). Mean OT concentrations were barely above the detection limit of the assay (0.8 pg/ml) and mean PGFM concentrations were also low. There was no significant relationship between mean UCA scores and mean OT and PGFM concentrations. UCA was significantly higher ($P < 0.001$) in the 10 resistant estrous mares than in the other groups of mares. There was no significant difference between mean UCA scores of resistant and susceptible mares in diestrus or susceptible mares in estrus (Fig. 1). In estrus, the administration of OT caused a significant decrease ($P < 0.05$) in UCA scores in both groups of mares (Fig. 2). UCA scores returned to baseline levels significantly faster ($P < 0.05$) in resistant (6 min) than in susceptible (8 min) mares after OT administration. In diestrus, the administration of OT did not have any significant affect on UCA.

4. Discussion

Ultrasonography is a technique available to all veterinary practitioners and is a relatively non-invasive means of assessing uterine contractility. No OT release was

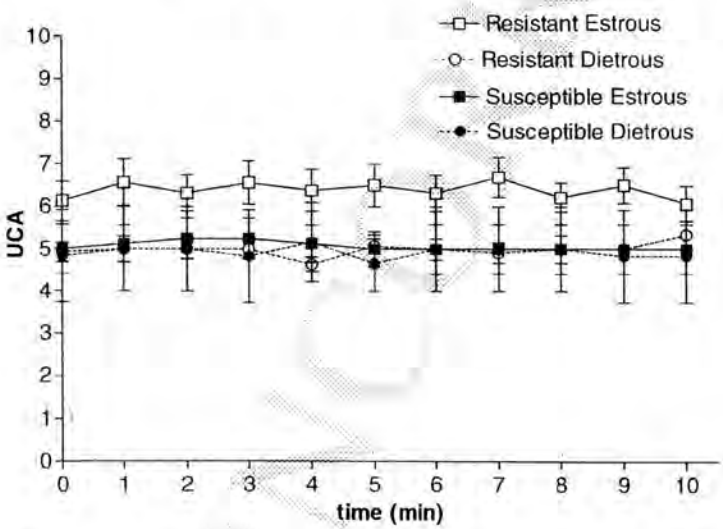

Fig. 1. Uterine contractile activity in resistant and susceptible mares in estrous and diestrous.

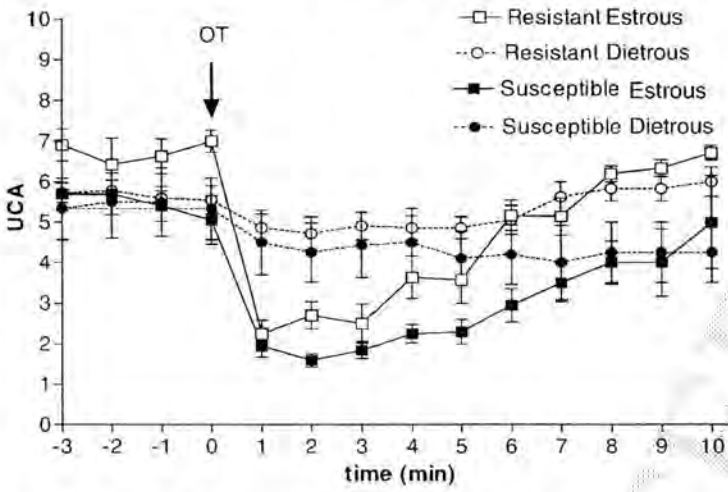

Fig. 2. Uterine contractile activity before and after OT administration (1 IU/20 kg) to resistant and susceptible mares in estrous and diestrous.

observed as a result of ultrasonography and PGFM levels remained at concentrations barely above the detection limit of the assay. This strongly suggests that transrectal ultrasonography has very little effect on uterine function. In the present study, a significant reduction in UCA scores was visually observed within 1 min of OT administration in both resistant and susceptible estrous mares. This further confirms the ability of OT to enhance impaired UCA and increase uterine clearance, within 8 min from OT administration, in mares susceptible to PMIE as shown previously [3]. Uterine spasm, the ecbolic effect of OT on myometrial function, reduced UCA scores and was seen after intravenous OT administration as a shortening of the vertical uterine axis combined with a visual decrease in UCA. OT administration on Day 7 of diestrus failed to elicit statistically significant changes in UCA in both groups of mares probably due to high progesterone concentrations. Diminished PGFM response to OT administration has been shown from as early as Day 2 of diestrus compared to the day of ovulation [4]. Resistant mares had significantly higher UCA scores in estrus than on D7 of diestrus. Although direction and speed of the uterine contractions were not evaluated in the present study, rapid changes in these parameters could clearly be seen in the uterine peristaltic motion of estrous mares but not in diestrous mares. In diestrus, uterine peristaltic motion was more uniform and individual "waves" were not as discernible as in estrus. This corresponds to results on uterine electrical activity which show that electrical activity bursts are of longer duration in diestrus but occur more frequently and are of shorter duration in estrous mares [5]. Impaired UCA is thought to contribute to the inability of the susceptible uterus to clear intrauterine fluid accumulations [5]. In the present study, we showed that estrous mares susceptible to PMIE had significantly lower baseline UCA than genitally normal mares. We concluded that transrectal ultrasonography was a useful tool for monitoring and evaluating UCA and did not appear to stimulate ecbolic hormone release.

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Plasma concentrations of 13,14-dihydro-15-ketoprostaglandin $F_{2\alpha}$ in mares during uterine involution

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Summary: *Twelve mares were allowed to foal naturally, after which they were monitored to study uterine involution. Starting on day 3 after parturition, the internal genital tract was examined per rectum manually and ultrasonographically every other day for changes in uterine characteristics and ovarian activity. By day 5, gravid and nongravid uterine horns were similar in size, and by day 7, uterine fluid was absent. On day 7 after parturition, endometrial biopsy samples were obtained for histologic evaluation, and uterine swab specimens were obtained for microbiologic culture. Uterine swab specimens from 10 of 12 mares had slight bacterial growth. The uteri of 8 of the 12 mares were histologically involuted by day 7. All mares ovulated 7 to 12 days after parturition. Concentrations of 13,14-dihydro-15-keto-PGF$_{2\alpha}$ (PGFM) were measured in jugular plasma samples obtained daily for 21 days after parturition. Concentrations of PGFM were low by the day after parturition, and there was no significant correlation between uterine involution and PGFM concentrations in these mares. All 12 mares were bred at the first estrus after parturition, and 9 became pregnant.*

Unlike other domestic species, mares usually are ready to breed and establish pregnancy within 10 days after parturition. With normal passage of the placenta, little damage occurs to the endometrium because of the epitheliochorial type of placentation in mares. Uterine involution in mares occurs quickly, with rapid loss of the microcaruncles and decrease in size of the endometrial glands.[1,2] Release of prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) from the uterus is an integral factor in the events preceding and following parturition; however, there is little detailed information on concentrations of $PGF_{2\alpha}$, measured as the blood metabolite 13,14-dihydro-15-keto-$PGF_{2\alpha}$ (PGFM), between parturition and the first estrus after parturition in mares. In one study,[3] blood was collected from 3 normally foaling mares for 2 to 4 days after parturition, and concentrations of PGFM in plasma were found to be low by the second day, with further release of $PGF_{2\alpha}$ on day 3.

In cows, there is a positive correlation between the duration of high concentrations of PGFM in plasma after parturition and the time required for completion of uterine involution.[4] Furthermore, administration of high doses of $PGF_{2\alpha}$ in cows hastened uterine involution.[5] From these results, it was postulated that $PGF_{2\alpha}$ exerted an apparently beneficial effect on uterine involution by improvement of myometrial tone. It is not agreed whether $PGF_{2\alpha}$ has an effect on myometrial contraction in mares,[6,7] but one study reported an increased foaling rate in mares that received injections of a $PGF_{2\alpha}$ analogue from foaling until the first estrus after parturition.[8] The purpose of the study reported here was to investigate uterine involution in normally foaling mares and to determine the concentrations of PGFM in plasma during the portpartum period.

Materials and Methods

Animals—Pregnant mares (n = 12), 4 to 18 years old and weighing 400 to 550 kg, with known breeding dates, were closely observed for signs of impending parturition. Day of foaling was designated day 0. Time of foaling, length of third-stage labor, weight of foals, and weight and condition of placentae were recorded. Mares were maintained on pasture during the day and kept in box stalls at night.

Postparturient sampling—A jugular plasma sample was obtained immediately after parturition and twice daily thereafter for 21 days. Samples were frozen at −20 C until analyzed for concentrations of PGFM, using a previously validated radioimmunoassay.[9] Values greater than the mean ± 2 standard deviations were considered to be increased above baseline.

The internal genital tract was examined by manual palpation and ultrasonography per rectum every other day from day 0 to 21. The diameter of

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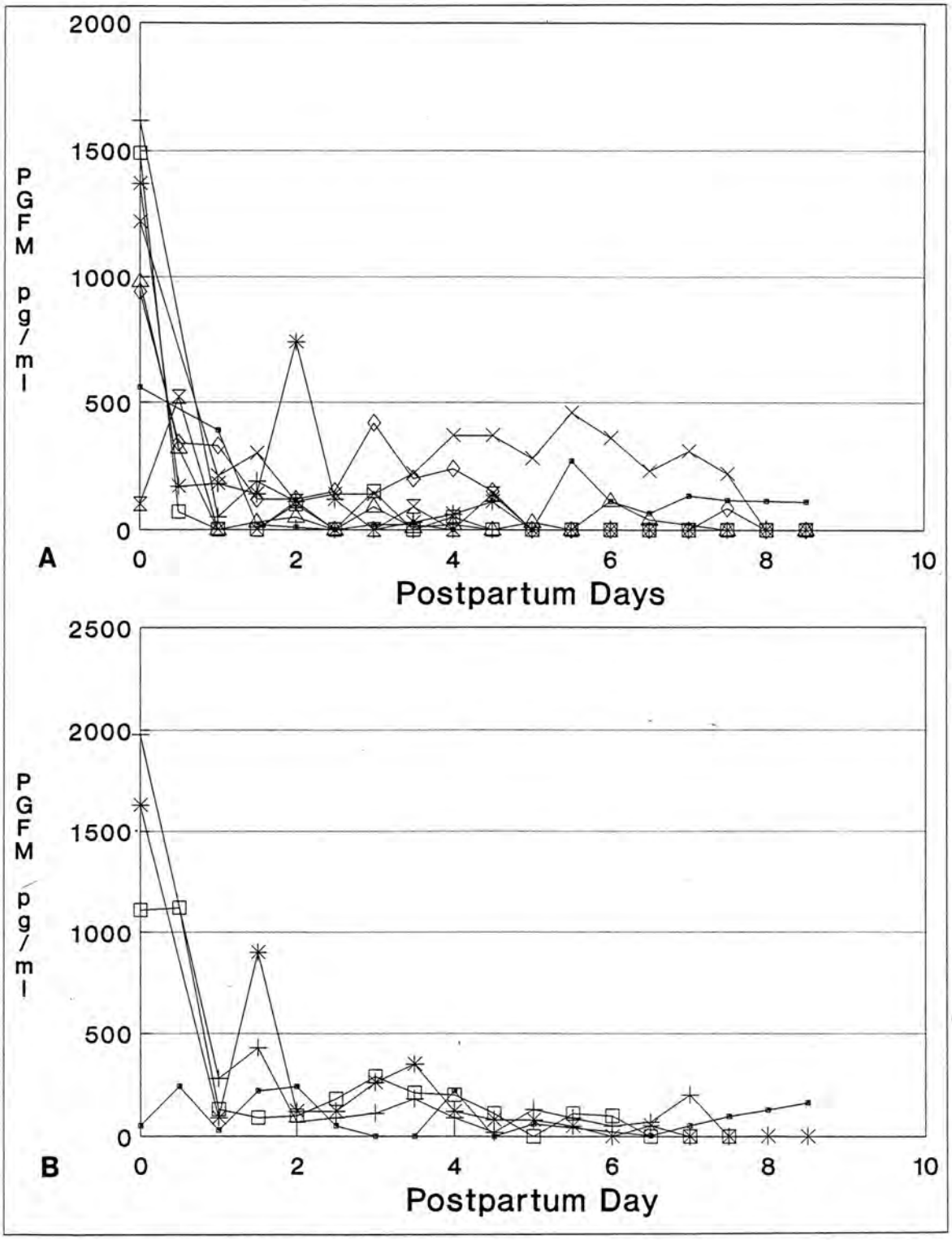

Figure 1—Concentrations of 13,14-dihydro-15-ketoprostaglandin $F_{2\alpha}$ (PGFM) in plasma on postpartum days 0 to 8. (A) Graph for 8 mares in which endometria were involuted at day 7 after parturition. Each line represents 1 mare. (B) Graph for 4 mares in which endometria were not involuted at day 7 after parturition. Each line represents 1 mare.

the base of each uterine horn, presence of fluid, any uterine abnormality, ovarian activity, and cervical changes were recorded.

To characterize cell content of postpartum vulvar discharge, swab specimens were obtained daily from the vaginal vestibule for the first 7 days after parturition and were stained[a] for cytologic evaluation. Specimens were subjectively classified on a scale of 0 to +3 according to quantities of RBC, neutrophils, and epithelial cells. On day 7, a uterine swab specimen and endometrial biopsy sample were obtained. The uterine swab specimen was cultured aerobically on sheep RBC agar and MacConkey agar at 37 C for 48 hours. Endometrial biopsy samples were evaluated histologically for involution by criteria previously reported.[1,2]

Breeding—All mares were bred once or twice during the first estrus after parturition and, if not determined to be pregnant by ultrasonography, were bred again at the subsequent estrus.

Statistical analysis—The difference in diameter between gravid and nongravid uterine horns was evaluated for each day by paired *t* test, and $P < 0.05$ was considered significant.

Results

Foaling—Twelve live foals were delivered. Ten of the mares foaled without complications. One mare required simple traction and rotation of the foal to relieve hiplock. Another mare exhibited strong first-stage labor for 1.25 hours without progressing into second stage and was given 10 IU of oxytocin. The foal was delivered without further assistance within 10 minutes.

Mean length of third-stage labor for all 12 mares was 1.3 ± 1.9 hours. Mean weight of the foals was 49 kg (range, 43.6 to 59), and mean weight of the placentae was 5.6 kg (range, 4.0 to 7.0). All placentae appeared normal on gross examination.

Concentration of PGFM—Concentrations of PGFM in plasma decreased to < 460 pg/ml in 10 of the mares (including the mare that received oxytocin at parturition) by day 1 after parturition. The concentration for 1 mare was 740 pg/ml at day 2. Concentrations for another mare were high on day 2 but decreased to low values by day 3. After day 3, the concentration of PGFM was baseline in all mares for the remainder of sampling. For 8 of the 12 mares, the endometrium was histologically involuted at day 7. Differences did not exist between mares that had involuted endometria on day 7 and those that had noninvoluted endometria on day 7 in concentrations of PGFM in plasma for days 0 through 21 (Figure 1 shows first 8 days).

Physical findings—For all mares, the cervix remained open until after the first postpartum ovulation, which occurred between 7 and 12 days (mean = 9.25) after parturition. All but one mare had a small amount of nonechogenic fluid in the uterine lumen at some time during the first 6 days after parturition. By day 7, uterine fluid was no longer detectable ultrasonographically in any mare. None of the mares received intrauterine or other medical treatment prior to breeding at the first estrus after parturition. Both uterine horns decreased gradually in diameter between days 0 and 15. Only at day 3 were the previously gravid and nongravid horns significantly different in size (Table 1). At day 5 and thereafter, gravid and nongravid horns did not differ in size ($P > 0.05$).

Table 1—Mean diameters of the previously gravid and nongravid uterine horns measured at the base days 3 through 21 after parturition in 12 mares

Day	Gravid horn (mm [+/−SD])	Nongravid horn (mm [+/−SD])
3	91.2 (20.1)*	74.9 (23.5)
5	76.7 (21.6)	64.4 (13.8)
7	66.1 (9.1)	65.5 (10.0)
9	61.2 (11.5)	66.2 (9.7)
11	64.1 (10.5)	64.2 (15.1)
13	59.8 (14.6)	59.0 (5.9)
15	50.6 (7.5)	55.1 (10.9)
17	53.7 (7.5)	52.5 (9.0)
19	57.8 (5.0)	53.0 (8.8)
21	48.6 (7.1)	49.7 (6.1)

*Difference at the $P < 0.05$ (paired *t* test) level between gravid and nongravid horn.

Vestibular cytologic findings—During the first 7 days after parturition, the predominant cell types seen in swab specimens from the vaginal vestibule were neutrophils and epithelial cells. In swab specimens from 11 of the mares, few RBC were found after day 3.

Cultures of uterine swab specimens—Day-7 uterine swab specimens from 10 of the 12 mares yielded a few colonies of microorganisms. The organisms isolated included β-hemolytic streptococcus, *Escherichia coli*, *Actinobacillus* spp, or *Corynebacterium* spp.

Endometrial histologic findings—For 8 of the mares, the endometrium was classified as involuted on the basis of results of histologic examination on day 7 after parturition. For these 8 mares, the microcaruncles were no longer present, the luminal epithelium was intact, and the endometrial glands were no longer distended. Foci of hemosiderophages were at the sites of the microcaruncles.

Subsequent breeding—Nine mares became pregnant when bred at the first estrus after parturition, 2 mares became pregnant when bred at the subsequent postpartum estrus, and 1 mare did not become pregnant that season. There appeared to be no relationship between presence of neutrophils in vestibular swab specimens and conception when the mare was bred at the first estrus after parturition.

Discussion

The rate of involution in the mares of this study was rapid after normal foaling, and plasma con-

[a]DiffQuik, American Scientific Supplies, McGaw Park, Ill.

centrations of PGFM decreased quickly, with only small increases in some mares after day 1.

Our histologic endometrial findings agreed with those of previous histologic and ultrastructural studies, which have shown that the endometrium of mares is repaired by day 7 after parturition.[1,10] This early recovery of the endometrium apparently results from the relatively noninvasive nature of the equine placenta and may account for the high conception rates achieved from breedings at the first estrus after parturition in this and other studies. Reduction in diameter of the uterine horns also occurs rapidly after foaling and, in our study, appeared to be complete by day 15 to 21, in agreement with findings of other studies.[1,11]

In cows, release of $PGF_{2\alpha}$ from the uterus is believed to reflect uterine damage and/or repair.[4] If the same were true in mares, it would be normal for release of $PGF_{2\alpha}$ from the mare uterus to be relatively short-lived after uncomplicated foaling.

There appeared to be no relationship between vestibular cytologic findings and uterine involution or conception rates at the first estrus after parturition, although one mare that did not conceive when bred at the first estrus after parturition still had many (3+) RBC in the vulvar discharge by day 7. This mare may have had greater endometrial damage at parturition than the other mares. The change over time of neutrophil numbers obtained on vestibular swab specimens appeared to be similar to that found in another study[12] in which the endometrium was swabbed during the postpartum period. Also in agreement with findings of our study, Koskinen and Katila[12] found that the presence of neutrophils had no effect on fertility of mares bred at the first estrus after parturition.

Consistent with other reports,[1,12] bacteria, most frequently β-hemolytic streptococci, were isolated from the postpartum uterus of the mares in our study. In at least 9 of the mares, these bacteria were presumably eliminated by the time the embryo entered the uterus.

Uterine fluid was commonly detected by ultrasonography during the first 6 days after parturition. McKinnon et al[11] similarly reported fluid accumulations in the postpartum uterus that decreased after postpartum day 5. Uterine fluid was not detectable in any of the mares in our study by the time of the first postpartum ovulation. Uterine fluid accumulations at this time have been associated with significantly decreased conception rates.[11]

We found that, of the 4 mares for which the uterus was not involuted at day 7, 2 did and 2 did not become pregnant when bred at the first estrus after parturition. There was no difference in the time interval between parturition and ovulation among these 4 mares that did and did not become pregnant (7 and 11 days, 8 and 10 days, respectively). In mares, ova are fertilized in the oviduct, and the embryo does not reach the uterine lumen until 5 to 6 days after ovulation. It may not be necessary for uterine involution to have occurred at the time of fertilization, provided it is complete by the time the embryo reaches the uterine lumen.

Concentrations of PGFM were low by the day after parturition, and there appeared to be no significant correlation between uterine involution and concentrations of PGFM in the plasma of the mares in our study. We suggest that this study be expanded to include subfertile mares or mares with periparturient problems (ie, retained placenta, dystocia, cesarean section) that might affect uterine involution.

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Immunolocalization of oxytocin and neurophysin in the mare uterus

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The presence of oxytocin in the mare uterus was determined by radioimmunoassay of tissue extracts and uterine lavage fluid, and by immunohistochemistry. Samples were obtained from mares during the oestrous cycle and on day 14 of pregnancy. Immunoreactive oxytocin was detected in extracts of endometrium and myometrium, and in uterine lavage fluid. In tissue sections, the luminal epithelium, the epithelium of the superficial, but not deep, glands, blood vessel walls and myometrium all stained positively for oxytocin, whereas only the luminal epithelium and epithelium of the superficial glands stained positively for neurophysin. These results indicate that oxytocin is synthesized in the epithelium but not in the myometrium. Immunostaining for oxytocin was most intense in early pregnancy and least intense during mid- to late dioestrus. The great variation in staining density during different reproductive states indicates that uterine oxytocin may have an autocrine or paracrine role in controlling uterine function.

Introduction

Plasma concentrations of oxytocin in mares have been described variously as high in oestrus and early dioestrus (Burns *et al.*, 1991), high in late dioestrus (Tetzke *et al.*, 1987) or as low throughout the oestrous cycle (Stevenson *et al.*, 1991). It is generally considered that oxytocin is synthesized and secreted with its neurophysin from the hypothalamus and posterior pituitary. However, in other species, it is becoming apparent that synthesis of neurohypophyseal hormones is not confined to the classical hypothalamo–neurohypophyseal axis. Oxytocin is known to be present in the ovary (Wathes and Swann, 1982), testis (Nicholson *et al.*, 1984), placenta (Makino *et al.*, 1983) and uterus (Ciarochi *et al.*, 1985), and in some non-reproductive sites (for review see Wathes, 1989).

Oxytocin-encoding mRNA has been reported in the uteri of rats (Lefebvre *et al.*, 1992), women (Chibbar *et al.*, 1993), sows (Boulton *et al.*, 1996) and mares (Behrendt *et al.*, 1997), but not pregnant ewes (Wathes *et al.*, 1996). The role of uterine oxytocin remains unclear, but it may have a paracrine action on endometrial prostaglandin synthesis (Lefebvre *et al.*, 1992).

There is very limited information in any species on localization of oxytocin to specific cell types within the uterus. In rats, immunostaining indicates that most of the peptide is present in the epithelium during pregnancy, whereas in non-pregnant rats very little staining for oxytocin was observed in these cells (Lefebvre *et al.*, 1992). In sows, *in situ* hybridization localized mRNA encoding oxytocin mainly to the luminal epithelium and muscle layers (Boulton *et al.*, 1996); the concentration of mRNA encoding oxytocin varied with the stage of the oestrous cycle.

In the present study, the presence of immunoreactive oxytocin in the mare uterus was determined by radioimmunoassay of extracts of uterine tissue and lavage fluid, and the density and distribution of oxytocin in the mare uterus during the oestrous cycle and early pregnancy was determined by immunostaining. From what is known about synthesis of neurophypophyseal peptides via prohormones, it is accepted that the presence of neurophysins outside the

neurohypophysis indicates secretion of neurohypophyseal hormones outside the supraoptico-hypophyseal system (Ivell *et al.*, 1983). In the present study therefore, immunoreactive neurophysin was localized within the uterus of mares to determine whether oxytocin was synthesized at this site.

Materials and Methods

Animals

Uterine samples were collected *post mortem* from eight mares of mixed breeding, aged 17–25 years. Each mare was classified as being in oestrus, early dioestrus, or mid- to late dioestrus, according to the presence and size of ovarian structures. Plasma progesterone assays confirmed the cycle stage. Samples were also collected *in vivo* from a further 11 mares aged 8–17 years, weighing 300–400 kg. In these 11 mares, the stage of cycle was monitored by transrectal ultrasonography of the ovaries each day until the day of ovulation, which was designated as day 0. Three of these 11 mares were inseminated for collection of samples at day 14 of pregnancy. Pregnancy was confirmed by ultrasonography.

Uterine lavage

Uterine lavage fluid was collected from the 11 mares (six in oestrus, five on day 7 dioestrus and from two of the mares on day 14 of pregnancy) by infusing 60 ml sterile phosphate buffered saline (pH 7.4) into the uterus and recovering it via gravity. The lavage fluid was centrifuged at 3000 *g* for 10 min at 4°C and the supernatant was stored at –70°C for oxytocin assay. Each sample was assayed separately.

Collection of uterine tissue

Complete sections of uterine horn were obtained from mares at an abattoir and were embedded in OCT compound (Miles Inc, Elkhart, IN) before being snap-frozen in a slurry of isopentane and dry ice. The samples were stored at –70°C for immunohistochemistry. In further samples, the endometrium and myometrium were separated and frozen separately at –70°C until extraction and assay for oxytocin. Transcervical endometrial biopsies were obtained from nine of the cyclic mares. Samples were collected from the three pregnant mares on day 14 of pregnancy. The biopsies were prepared for immunohistochemistry as described above.

Immunohistochemistry

Tissue sections (7 µm) were immunostained using an avidin–biotin complex method (Watson and Thomson, 1996). Two primary anti-oxytocin antibodies were used initially (UCB A481/R4V; Accurate Chemical and Scientific Co, Westbury, NY; and O3882; Sigma Chemical Co, Poole) at 1:1250 and 1:50, respectively. Both of these antibodies had minimal crossreactivity with other known neuropeptides and pituitary hormones. Staining patterns were identical with both antibodies, but the staining was stronger with UCB A481/R4V; therefore, this antibody was used for subsequent sections. The anti-neurophysin antibody (NCL-NP_{p1}, Vector Laboratories, Peterborough) was used at a dilution of 1:1000. Only selected representative sections were stained for the presence of neurophysin.

Controls to test the specificity of staining included replacement of the primary antibody with normal horse serum or an anti-$CD4^+$ antibody, and preabsorption of the 1:1250 dilution of the antiserum with 250 µg oxytocin ml^{-1} for 24 h at 4°C. Sections of horse posterior pituitary were included as positive tissue controls.

Evaluation of staining

Staining of uterine sections was graded from 0 to 5, where 0 represented no staining and 5 was the most intense. Each section was graded for six different areas where present: luminal epithelium, superficial endometrial glands, deep endometrial glands, stroma, myometrium and blood vessels.

Progesterone assay

Progesterone concentrations were measured in unextracted plasma using a technique originally reported by Corrie *et al.* (1981) and modified by Law *et al.* (1992). The antiserum was donated by the Scottish Antibody Production Unit, Carluke. The main crossreactivities of the antiserum were with 5-pregnane-3,20 dione (9.5%), 11-deoxy corticosterone (6.2%) and 17-hydroxyprogesterone (3.4%). Progesterone standards were prepared in ovariectomized mare plasma. Assay sensitivity was 0.5 ng ml^{-1} and intra- and inter-assay coefficients of variation were 9.0% and 12.6%, respectively.

Oxytocin assay

The tissues were extracted as described by Wathes *et al.* (1986), by homogenization and boiling in acid. The supernatant was freeze-dried, reconstituted in 0.7 ml oxytocin assay buffer (2.56 g barbituric acid l^{-1}, 8.28 g sodium chloride l^{-1}, 0.016 g L-cysteine l^{-1}, 3.72 g EDTA l^{-1} and 2.0 g bovine gamma globulin l^{-1}, made up in distilled water, pH 9.0) and extracted using C^{18} SepPak cartridges (Waters Associates, Millipore Co, Milford, MA). The radioimmunoassay of the tissue extracts and the extracted uterine lavage fluid were performed as described by Thornton *et al.* (1986), with an antiserum characterized by Sheldrick and Flint (1981,1986). The detection limit for the assay was 0.8 pg ml^{-1} and the intra- and inter-assay coefficients of variation were 7.5% and 11.1%, respectively.

Statistical analyses

A one-way analysis of variance was used to detect differences due to reproductive state and least significant difference mean comparisons were performed where appropriate.

Results

Lavage fluid

Oxytocin was detected in uterine lavage fluid from oestrous (44.2 ± 17.7 pg 60 ml^{-1}) and dioestrous (48.6 ± 16.7 pg in 60 ml) mares, and concentrations were not significantly different. Oxytocin concentrations in lavage fluid from the two pregnant mares sampled were lower (14 and 18 pg in 60 ml) than in any of the cyclic mares.

Endometrial tissue

Oxytocin was detected in approximately equal amounts in extracts from endometrium (267.9 ± 38.6 pg g^{-1} tissue) and myometrium (270.3 ± 44.8 pg g^{-1} tissue). An insufficient number of samples was obtained from oestrous mares to compare differences between cycle stages.

Immunohistochemistry

In all uterine sections, strong positive immunostaining for oxytocin was visible in the luminal epithelium and in the epithelium of the superficial glands. The staining was discrete within the epithelial cells and tended to be located near the apical border. No staining was present in the deep

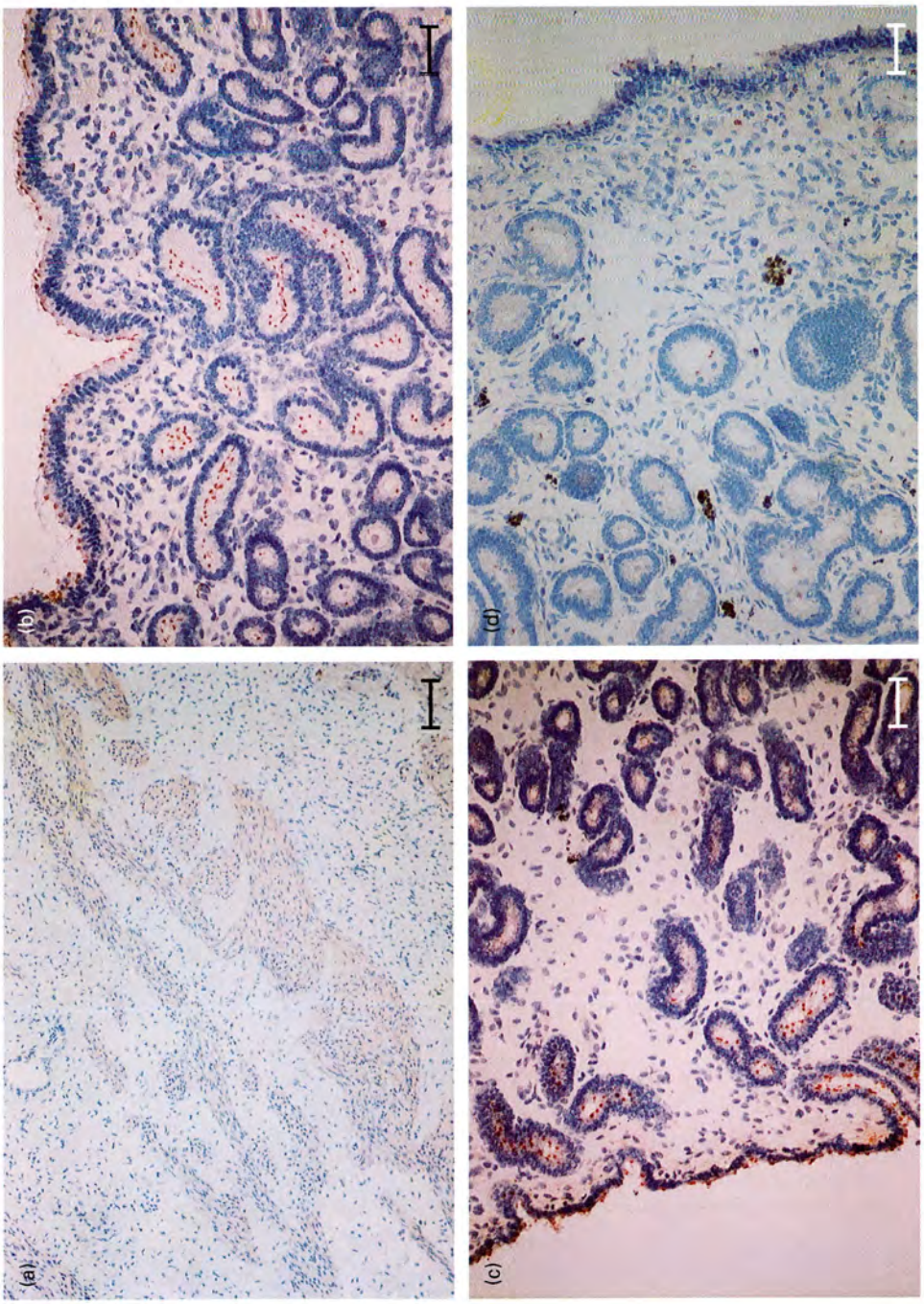

Fig. 1. Immunolocalization of oxytocin in the mare uterus, using 3-amino-9-ethylcarbazole as the chromagen (red). Sections were counterstained with Meyer's haematoxylin (blue). Immunostaining for oxytocin in: (a) the myometrium, scale bar represents 10 µm; (b) endometrium on day 14 of pregnancy, scale bar represents 5 µm; (c) endometrium of an oestrous mare, scale bar represents 5 µm; and (d) endometrium in mid to late dioestrus, scale bar represents 5 µm. The stained clumps of cells in this section are siderophages that have taken up the chromagen non-specifically. Haemosiderophages are common in parous mares (d), but not in nulliparous mares (a,b,c).

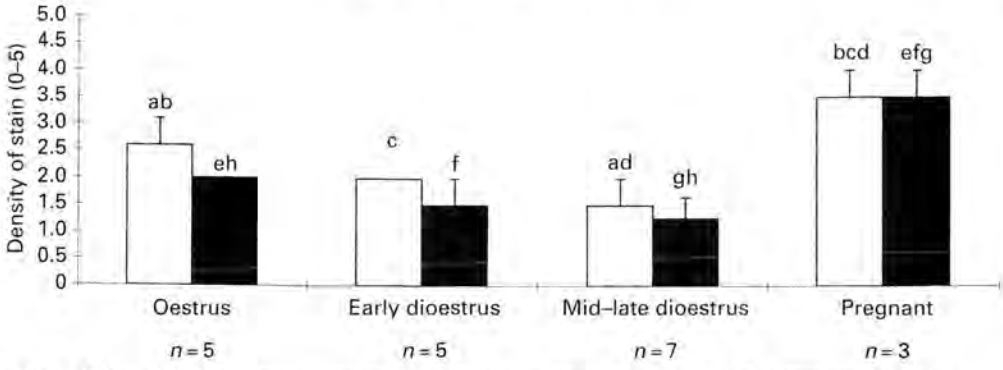

Fig. 2. Effect of oestrous cycle stage or pregnancy in mares on the intensity of immunostaining for oxytocin in the superficial endometrial glands (□) and luminal epithelium (■). Columns with the same letter are significantly different: a,b,h ($P < 0.05$); c,e ($P < 0.01$); d,f,g ($P < 0.001$).

glands or in the endometrial stroma. There was diffuse positive staining in well-defined regions of the myometrium (Fig. 1a). In some, but not all, mares, blood vessel walls showed positive staining.

Stage of cycle and pregnancy had a significant effect ($P < 0.01$) on the intensity of immunostaining for oxytocin (Fig. 2). Staining was most intense in pregnancy (Fig. 1b) and oestrus (Fig. 1c), whereas staining was least intense in mid- to late dioestrus (Fig. 1d). Staining of blood vessels did not appear to vary with cycle stage. Only the tissues obtained from the abattoir had myometrium attached. In these tissues, the myometrial staining was significantly more intense ($P < 0.01$) at oestrus than at dioestrus. Immunostaining for neurophysin was present in luminal epithelium and the epithelium of the superficial glands (Fig. 3a). The location of the staining within the cells was the same as for oxytocin. However, staining was not present in muscle or blood vessel walls. Staining was not observed in negative control sections of uteri (Fig. 3b). Positive staining was present in the nerve terminals of the neurohypophysis.

Discussion

The results of the present study indicate that oxytocin and its neurophysin are present in the uteri of cyclic and early pregnant mares. The distribution of immunostaining, with staining being most intense in the epithelium, supports observations in rats (Lefebvre *et al.*, 1992) and *in situ* hybridization results for mRNA encoding oxytocin in sows (Boulton *et al.*, 1996). The detection of mRNA encoding oxytocin in equine uteri (Behrendt *et al.*, 1997) indicates that at least some of the peptide identified by immunostaining was synthesized in the uterus. Furthermore, although some of the epithelial oxytocin may have been bound to specific receptors, the density of staining reported in the present study, with highest density in early pregnancy and at oestrus, does not correspond to concentrations of oxytocin receptors in the mare endometrium. Receptor concentrations are reported to be lowest during oestrus and highest in nonpregnant mares on day 14, and are significantly reduced on day 14 of pregnancy (Sharp *et al.*, 1997). Neurophysin is the conventional carrier protein for oxytocin. In the hypothalamo–neurohypophyseal system, oxytocin and oxytocin–neurophysin are produced from a common precursor molecule (Land *et al.*, 1983). In other studies, the simultaneous presence of immunoreactive oxytocin and its neurophysin has been interpreted as an indication of their synthesis within that tissue (Guldenaar *et al.*, 1984; Ciarochi *et al.*, 1985; Veeramachaneni and Amann, 1990). Therefore, the presence of both peptides within the epithelium indicates that oxytocin is synthesized at that site, whereas the lack of positive staining for neurophysin in muscle and blood vessel walls indicates that the oxytocin is receptor-bound, rather than being synthesized. In sows, mRNA encoding oxytocin was found in the myometrium (Boulton *et al.*, 1996) but the presence of the peptide was not investigated.

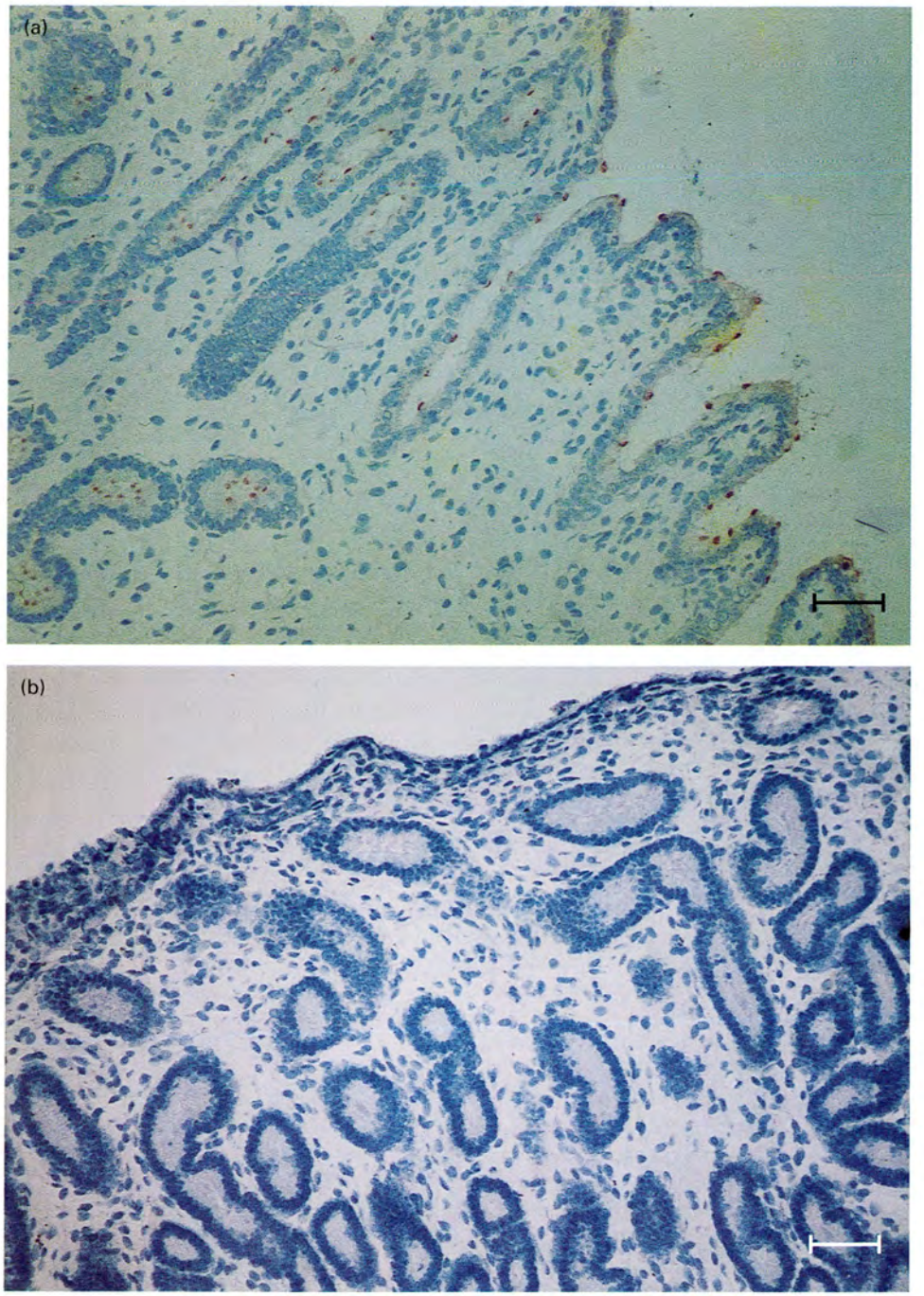

Fig. 3. Immunostaining of neurophysin in mare tissues: (a) the endometrium, scale bar represents 5 μm; and (b) negative control section in which antiserum was substituted with non-immune serum, scale bar represents 5 μm.

The discrete nature of the staining within the epithelial cells was unusual and indicates that the oxytocin and neurophysin may be contained within vesicles. The locations near the apical border of the cells may indicate that if these peptides are being manufactured in these cells, secretion is being directed into the lumen of the uterus and glands. This is in agreement with the observation that oxytocin is present in uterine flushings from the mares in the present study. However, there is a precedent for location of these peptides within vesicles. Within the hypothalamo–neurohypophyseal system, the precursor protein is translated from mRNA in the cell body of the neurone. The precursor is packaged into a secretory vesicle, which acts as a transport vehicle during axonal transport to the nerve terminals. Within these vesicles the endoproteolytic cleavages that excise the peptides from the precursor occur (Gainer *et al.*, 1985). Thus, in the endometrial epithelial cells, the precursor may be manufactured within the cytoplasm, but the peptides may be present, after cleavage, in secretory vesicles only.

Uterine oxytocin may have an autocrine action on epithelial prostaglandin synthesis and may affect myometrial contractility (Lefebvre *et al.*, 1992). In mares, immunostaining was lowest in mid- to late dioestrus, when the potential for synthesis of endometrial $PGF_{2\alpha}$ is highest. Oxytocin is thought to have a role in luteolysis in mares as administration of oxytocin in mid-dioestrus shortened the duration of the cycle (Goff *et al.*, 1987). These authors proposed that oxytocin of pituitary origin may be involved. It is possible that oxytocin of uterine origin may play a role and the low intensity of immunostaining at this time may reflect an increase in uterine secretion.

The high intensity of immunostaining for oxytocin during oestrus may indicate a role for uterine oxytocin in uterine immune defence at a time when the cervix is relaxed and the uterus prone to infection. Uterine contractility is known to be an important mechanism for expulsion of infection (Troedsson *et al.*, 1993) and therefore high local concentrations of oxytocin from local production may be situated ideally to stimulate myometrial contractions after intrauterine challenge. Chibbar *et al.* (1993) proposed that locally derived oxytocin may stimulate uterine contractions in pregnant women in the absence of changes in maternal plasma concentrations.

The high intensity of staining observed in early pregnancy was unexpected in view of the proposed role of oxytocin in luteolysis. However, high endometrial content of oxytocin and mRNA encoding oxytocin has been recorded in early pregnant sows (Trout *et al.*, 1995) and rats (Ciarochi *et al.*, 1985; Lefebvre *et al.*, 1992). In mares, continuous administration of oxytocin delayed luteolysis (Stout *et al.*, 1997) and administration of oxytocin to early pregnant mares failed to release $PGF_{2\alpha}$ (Goff *et al.*, 1987). Therefore, the presence of oxytocin is not inconsistent with luteal maintenance and pregnancy. In rats the amount of uterine oxytocin is increased by administration of oestrogen (Ciarochi *et al.*, 1985) and therefore it may be that the high concentrations of oestrogen produced by the equine conceptus (Zavy *et al.*, 1979) stimulate endometrial oxytocin production. Alternatively, the increased intensity of staining may reflect decreased oxytocin secretion, rather than increased production at a time when its normal secretion at the corresponding time during the oestrous cycle could result in luteal regression. This would explain the lower concentrations of oxytocin found in uterine lavage fluid from the two pregnant mares in the present study.

In conclusion, immunoreactive oxytocin is present in the mare uterus. The intensity of immunostaining varied with reproductive state, and was highest in early pregnancy. It is possible that endometrial oxytocin may have a paracrine or autocrine role in local control of uterine function.

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IMMUNOHISTOCHEMICAL LOCALISATION OF OXYTOCIN AND NEUROPHYSIN IN THE EQUINE ENDOMETRIUM USING TRANSMISSION ELECTRON MICROSCOPY

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Introduction

Recently it has been reported that oxytocin (OT) is present out with the hypothalamo-neurohypophyseal system, particularly within the ovary and uterus. It has been demonstrated that OT has been identified in the corpus luteum of cows, goats, sheep and women. However unlike other species the corpus luteum of the mare does not appear to be a source of OT. OT-mRNA has been identified in the equine endometrium[1] and our previous studies[2] have shown that OT and its carrier protein, neurophysin (NP), are present in the uterus of the cyclic and early pregnant mare.

The aims of this study were to localise OT and NP in the equine endometrium at the ultrastructural level.

Material and Methods

Samples of equine endometrium were obtained by transcervical biopsy from cyclic non-pregnant mare. The tissues were fixed in a mixture of 4% paraformaldehyde/ 0.1% glutaraldehyde in 0.1M sodium cacodylate buffer and were embedded in acrylic resin (UNICRYL). Ultrathin sections were cut and were immunostained using the avidin-biotin complex (ABC) method described previously[3] with 3'-diaminobenzidine (DAB). The primary antibodies were specific for either OT or NP. Sections were counterstained with 2% osmium tetroxide. Equine posterior pituitary gland was used as a positive control and negative controls were incubated in which the primary antisera were replaced with either buffer or normal rabbit serum.

Results and Discussion

The general staining for both OT and NP was similar. Positive staining was present in granular vesicles of the simple columnar luminal epithelium. Vesicles containing NP were slightly more electron dense than those stained for OT. In the superficial glands, positive staining was present in secretory vesicles of the columnar glandular epithelium, towards the centre of the uterine gland. Staining was absent in the endometrial stroma and mid- and basal gland regions and in the negative control sections. This study has identified the location of OT synthesis in the luminal epithelium and superficial glands of the equine endometrium and is the first detailed report on location of OT in the uterus of any species.

Further studies are needed to define the role of local endometrial OT synthesis in uterine function in the mare.

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Release of oxytocin and prostaglandin $F_{2\alpha}$ around teasing, natural service and associated events in the mare

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Abstract

Mating has been shown in many species to provoke the release of oxytocin (OT). In our study, various stimuli were applied to mares to study release of OT and prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) associated with mating. Blood samples were collected from mares around the time of teasing both in oestrus and dioestrus and at mating. For comparison, blood samples were also collected at the time of manual manipulation of the genital tract and after intrauterine infusion of 500 ml phosphate buffered saline (PBS). Additional samples were collected 16 to 18 h after mating. Mating caused a significant increase in OT in all mares and teasing caused a significant OT response in 6 of 10 oestrous and 3 of 5 dioestrous mares. However, mating and teasing had no significant effect on concentrations of 15-keto-13,14-dihydro-$PGF_{2\alpha}$ (PGFM). Manual manipulation of the clitoris, vagina and cervix caused significant OT release in all mares and intrauterine infusion of 500 ml PBS caused significant OT release in three of the five mares. However, only one mare had a significant $PGF_{2\alpha}$ response during manual manipulation and only one responded positively to intrauterine infusion of 500 ml PBS. We concluded that events around mating, including stimulation of the genital tract and uterine distension, often caused an increase in circulating concentrations of OT but only rarely in PGFM.

Keywords: Horse; Oxytocin; Prostaglandin; Mating; Teasing

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1. Introduction

Release of oxytocin (OT) can be elicited at oestrus by mating and presence of the male (McNeilly and Ducker, 1972; Gilbert et al., 1991) and these high concentrations of OT may serve to facilitate gamete transport (Wathes, 1984; Gilbert et al., 1992). The pattern of OT release at mating appears to be highly species-dependent. In some species the physical act of coitus is thought to be a relatively minor component in stimulating the release of OT (McNeilly and Ducker, 1972) and OT peaks may be provoked by the sensory and psychic stimuli occurring at the time of mating (Campbell and Petersen, 1953; McNeilly and Folley, 1970; McNeilly and Ducker, 1972). In the mare, mating is a complex combination of sensory, psychic and mechanical stimuli involving teasing, mounting, intromission and ejaculation by the stallion. It is known that teasing stimulates OT peaks in the mare at mating (Alexander et al., 1995), but there is only limited information on the pattern of OT release (Walmsley, 1963; Alexander et al., 1995).

Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) release is high in late dioestrus in mares, in association with luteolysis (Goff et al., 1984). Release of $PGF_{2\alpha}$ can also be stimulated at other stages of the cycle by OT (Goff et al., 1987) and there is a positive correlation between plasma OT concentrations and $PGF_{2\alpha}$ release in nonpregnant mares (Sharp et al., 1997). Therefore, any increase in OT at mating in mares might be accompanied by release of $PGF_{2\alpha}$. Both $PGF_{2\alpha}$ and OT have been implicated as ecbolic agents in mares (Troedsson et al., 1995). Persistent mating-induced endometritis is a major cause of subfertility in mares. Susceptibility to this condition is associated with low myometrial contractility (Troedsson et al., 1993). Increases in concentrations of $PGF_{2\alpha}$ and OT around the time of mating may therefore be important not only in gamete transport, but in stimulating uterine contractile activity after mating, which serves to evacuate collections of inflammatory uterine fluid and cellular debris in genitally normal mares (Hughes and Loy, 1975).

We hypothesised that mares susceptible to endometritis, with delayed uterine clearance after mating may have defective release of ecbolic hormones. However, there is no detailed information on endocrine events occurring around the time of mating and other intrauterine interventions in genitally normal mares. Therefore, in the present study, we measured the release patterns of the ecbolic hormones OT and $PGF_{2\alpha}$ at the time of mating in the mare and some hours following mating when clearance of uterine contamination may occur. We also investigated the effect of a range of stimuli associated with mating on OT and $PGF_{2\alpha}$ release.

2. Materials and methods

2.1. Animals

Eleven fertile mares, aged 5 to 15 years and weighing between 350 and 480 kg, were used. These mares had been classified as genitally normal, according to their reproductive history, negative endometrial cytology and culture, and endometrial biopsy scores of 1–2A (Kenney and Doig, 1986). Oestrus was detected by teasing with a stallion,

combined with transrectal ultrasonographic examination of the genital tract. When the mare responded positively to teasing and uterine oedema was present, with a follicle of at least 35 mm present on the ovaries, the mare was considered to be in oestrus. Ovulation was detected ultrasonographically by the disappearance of the follicle and the presence of a corpus haemorrhagicum on the ovary. Day of ovulation was designated as day 0. A stallion with good libido was used in experiments 1 and 2.

2.2. Experimental procedures

The same blood sampling protocol was used in all experiments. On the day of the experiment, an indwelling cannula (13 gauge, Presidio Medico, Ecouen, France) was placed in the jugular vein aseptically under local anaesthesia. Blood samples were collected at 2-min intervals for 30 min before, during, and 1 h after the application of the stimulus, and then for another hour, at 5-min intervals.

Five different stimuli were applied. In experiment 1, oestrous ($n = 5$) and dioestrous (day 7; $n = 5$) mares were teased by the stallion for 6 min (three samples) in teasing stocks. The stallion was allowed to nuzzle and nip the mare. In experiment 2, oestrous mares were teased as in experiment 1, but in this case teasing was followed immediately by mating ($n = 5$). In experiment 3, the genital tracts of oestrous mares ($n = 4$) were manually manipulated to simulate the individual stimuli associated with mating. All stimuli applied were exaggerated in duration in order to intensify the response to the particular stimulus. The mares' external genitalia were actively massaged for 6 min (three samples), while washing three times with tamed povidone iodine solution, after which the operator inserted a sterile gloved lubricated hand into the vagina, moving it carefully palindromically for 4 min (two samples), distending the vaginal walls without coming into contact with the cervix. The cervix was then massaged and manipulated for 2 min (one sample). In experiment 4, the mechanical effect that the introduction of fluid into the uterus and/or uterine distension has on hormonal release in oestrous mares ($n = 5$) was determined by intrauterine infusion of 500 ml phosphate buffered saline (PBS; pH 7.0). This high volume was chosen from a preliminary study in which cross-dimensional areas had been calculated from transrectal ultrasound scans of different volumes of infused buffer (Nikolakopoulos and Watson, 1999). It was seen that at this volume the fluid did not merely remain in one pocket, but distended and contacted a significant part of the endometrium (Nikolakopoulos, 1999). Additional blood samples were collected every 15 min between 16 and 18 h after mating for measurement of PGFM concentrations to determine whether the high uterine myoelectrical activity reported at this time after bacterial infusion in genitally normal mares (Troedsson et al., 1993) is due to the release of prostaglandins.

2.3. Sample handling

Blood samples were collected into evacuated heparinised tubes and placed on ice until separation. The samples were centrifuged at $2000 \times g$ for 15 min at 4°C. An aliquot of plasma was acidified with 10 M acetic acid (10 μl/ml plasma) for oxytocin assay. All samples were frozen at −70°C and subsequently stored at −20°C until assayed.

2.4. Oxytocin assay

Oxytocin was extracted from 2 ml plasma using C_{18} SepPak cartridges (Waters Chromatography, Milford, MA, USA) and the radioimmunoassay carried out in duplicate samples as described by Thornton et al. (1986) using a previously characterised antiserum (Sheldrick and Flint, 1981). The extraction recovery rate was 74.8%. The detection limit for the assay was 0.8 pg/ml. The intra- and inter-assay coefficients of variation were 7.5% and 11.1%, respectively.

2.5. 15-Keto-13,14-dihydro-$PGF_{2\alpha}$ assay

The release of $PGF_{2\alpha}$ was monitored by measuring its main initial plasma metabolite 15-keto-13,14-dihydro-$PGF_{2\alpha}$ (PGFM). Duplicate samples of unextracted plasma (0.2 ml) were assayed. Before the addition of antibody and radioactive tracer, 0.3 ml 0.25% bovine gamma globulin in buffer was added and the tubes were heat-treated for 30 min at 45°C. The antibody cross-reacted with 15-keto-$PGF_{2\alpha}$ (16.0%), 13,15-dihydro-$PGF_{2\alpha}$ (4.0%), and 15-ketodihydro-PGE_2 (1.7%). Other prostaglandins tested cross-reacted less than 0.1%. The detection limit of the assay was 20 pg/ml. The intra- and inter-assay coefficients of variation were 8.5% and 14%, respectively.

2.6. Statistical analysis

Mean baseline hormone concentrations were calculated from the average of the values obtained prior to the application of the stimulus (Time 0) and mean hormone concentrations were calculated for every 30-min interval thereafter. Concentrations below the detection limit of the assays were designated as equivalent to the detection limit of the respective assay. In the case of teasing and manipulation of the genital tract, mean stimuli values for both hormones were obtained from the samples corresponding to the time of the different stimuli application. During natural service and uterine infusion, mean OT values were obtained from the samples corresponding to the mean OT concentrations of the peak immediately after the application of the stimulus until OT concentrations returned to baseline levels. Mean baseline OT and PGFM concentrations from all experiments were compared using one-way ANOVA test. Mean baseline values and mean stimuli values for both OT and PGFM, for each experiment, were compared using a two sample *t*-test. In experiment 3, mean stimuli values for both OT and PGFM were compared with mean baseline values separately for each stimulus. The difference in magnitude of OT responses between different stimuli was compared using one-way ANOVA tests.

Because of the pulsatile nature of OT release and its short half-life, responses were assessed for individual mares. A mare was considered to have a positive OT response when the mean concentration of the peak immediately after the application of the stimulus exceeded the mean baseline concentrations + 2 × SD. Mean PGFM concentrations were calculated for each mare as above (30-min intervals) and a positive response

was recorded when the increase for each mare, at any 30-min interval after the stimulus application, exceeded the mean baseline concentrations + 2 × intra-assay coefficient of variation.

3. Results

3.1. Experiment 1

For the evaluation of responses to teasing, teasing periods before mating were also included. All oestrous teasings were accompanied by a display of oestrous behaviour such as posturing and clitoral winking; while during all dioestrous teasings, the mares demonstrated aggressive behaviour and rejected the stallion. Teasing caused a significant ($P < 0.05$) increase in OT concentrations in both oestrus and dioestrus (Fig. 1). There was no significant difference ($P > 0.1$) either between the number of mares responding to teasing with increased OT release in oestrus (6/10) and dioestrus (3/5) or in the magnitude of response. In both dioestrous and oestrous teasing periods, except those followed by mating, mean OT concentrations declined to baseline values within 30 min (Fig. 1). On three occasions, an OT peak was observed immediately prior to the initiation of teasing, coinciding with the entrance of the stallion into the mare's visual field. Although teasing had no significant effect on mean PGFM concentrations in oestrus (Fig. 1), elevations were observed in two of the five sampling periods after teasing. None of the dioestrous mares released significant amounts of $PGF_{2\alpha}$ at teasing (Fig. 1).

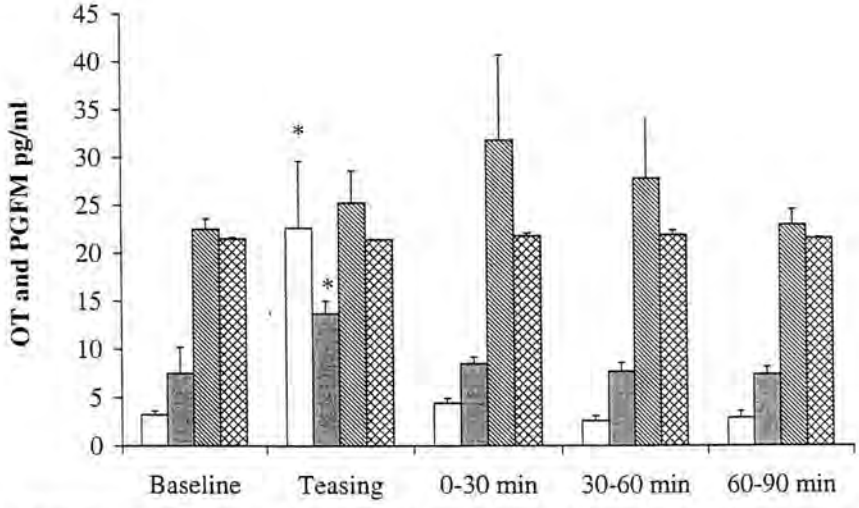

Fig. 1. Oxytocin (,) and PGFM (,) at teasing in 10 oestrous (,) mares and in 5 dioestrous (,) mares. Note that only 5 oestrous mares were sampled after teasing. Asterisks represent a significant increase from baseline concentrations (−30–0 min).

3.2. Experiment 2

All five mares had a positive OT response during natural service and mean stimuli values were significantly higher ($P < 0.05$) than baseline values (Fig. 2). Furthermore, in two mares in which false mounting occurred without intromission prior to successful mating, an oxytocin peak was observed. Mean OT concentrations after mating declined to baseline values within 30 min after the application of the stimulus. Mating had no significant effect on mean PGFM concentrations (Fig. 2), but minor elevations were observed in three of the five mares after mating. There was no detectable PGFM in the samples collected 16–18 h after natural service (data not shown).

3.3. Experiment 3

Manipulation of the genital tract consisted of three distinct stimuli to which the OT and PGFM response varied. Active massage of the clitoris and external genitalia significantly ($P < 0.05$) increased OT concentrations in two of the four mares. Both distension of the vaginal walls and cervical stimulation significantly increased ($P < 0.05$) mean OT concentrations. In only one of the mares did PGFM levels reach statistical significance when compared to mean baseline concentrations in response to manipulation of the genital tract. In all mares, mean OT concentrations returned to baseline levels within 1 h of the application of the stimuli (Fig. 3). Mean PGFM concentrations in the one responding mare did not return to baseline levels by the end of blood sampling, 2 h after the application of the stimuli.

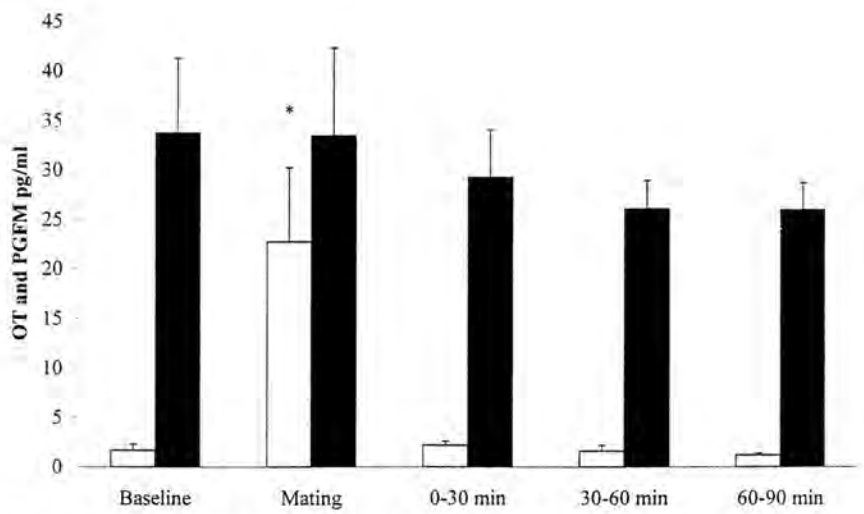

Fig. 2. Oxytocin (clear bars) and PGFM (shaded bars) in 5 oestrous mares around mating. Asterisk represents a significant increase from baseline concentrations (−30–0 min).

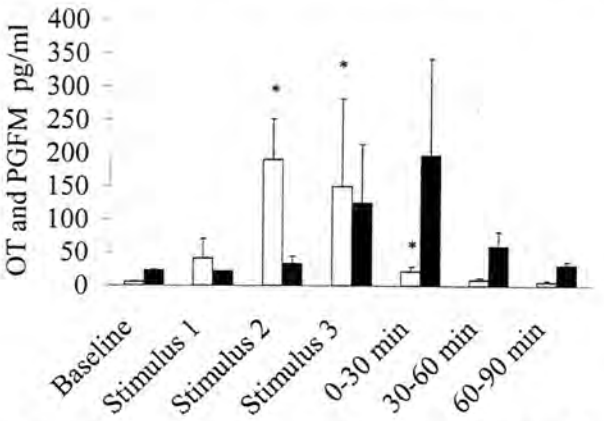

Fig. 3. Oxytocin (clear bars) and PGFM (shaded bars) levels around manipulation of the genital tract in 4 oestrous mares. Active massage of the vulva and clitoris (stimulus 1), distension of the vaginal walls (stimulus 2) and cervical manipulation (stimulus 3) are noted. Asterisk represents a significant increase from baseline concentrations (− 30–0 min).

3.4. Experiment 4

The intrauterine infusion of 500 ml PBS provoked a significant OT response ($P < 0.05$) (Fig. 4). Mean OT concentrations returned to baseline levels within 30 min from the time of infusion. There was a positive PGFM response in only one mare.

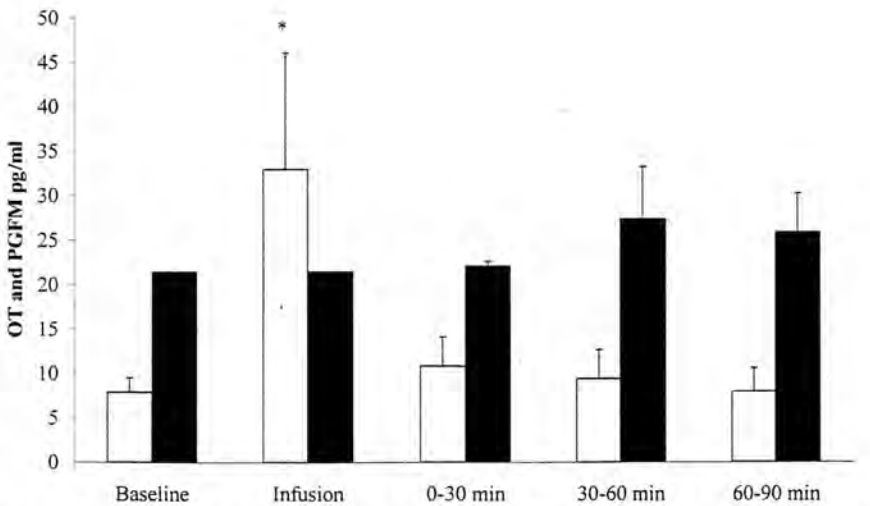

Fig. 4. Oxytocin (clear bars) and PGFM (shaded bars) in 5 oestrous mares after intrauterine infusion of 500 ml PBS. Asterisk represents a significant increase from baseline concentrations (− 30–0 min).

4. Discussion

In the present study, we have shown that numerous stimuli associated with mating can result in oxytocin release in genitally normal mares. The effect of central OT on reproductive behaviour has been reported to be species-specific and dependent on circulating concentrations of sex steroids (Caldwell et al., 1989; Carter, 1992; Insel et al., 1997). It has been shown in other species that external stimuli, such as olfactory (McNeilly and Folley, 1970; McNeilly and Ducker, 1972), visual (McNeilly and Folley, 1970; McNeilly and Ducker, 1972), and tactile (Schams et al., 1982) signals associated with teasing are directly responsible for the release of OT. In the mare, posturing is an indicator of oestrus and is an indispensable part of mating behaviour. It is provoked by visual, tactile and acoustical stimulation during teasing. In the present study, although oestrous teasing was associated with sexually receptive behaviour in all mares, OT peaks were detected in only some of the mares. In agreement with the present study, Madill et al. (1988) reported that not all oestrous mares released OT in response to teasing. It is possible however that peaks of OT were missed in the present study by sampling peripheral blood, as concentrations of OT are eight fold higher in blood collected from the intracavernous sinus of mares (Vanderwall et al., 1998). Exhibition of oestrus in the mare seems to be primarily dependent on the circulating concentrations of steroid hormones since, in our study, teasing at day 7 of dioestrus, when progesterone levels are high, provoked an OT response that was accompanied by rejection of the male. These observations support the concept that the steroid environment can modify responsiveness to somatosensory stimuli mediated by OT in the brain (Caldwell et al., 1996).

The physical stimulus of coitus per se is a relatively minor component in stimulating the release of OT in the oestrous goat (McNeilly and Folley, 1970; McNeilly and Ducker, 1972) and cow (Schams et al., 1982). By contrast, coitus-evoked OT release was reported in sows (Claus et al., 1989) and in women (Carmichael et al., 1987). It has been suggested that these inter-species differences are related to the degrees of physical stimulation caused by the male (Schams et al., 1982; Claus et al., 1989). The mating sequence in the mare includes a total mount time of 20 to 30 s with an insertion time before ejaculation of 10 to 15 s during which the stallion thrusts from six to nine times (McDonnell, 1992). In the mare, the oestrous cervix is relaxed, permitting dilation by the greatly distended glans penis, thus allowing intrauterine deposition of semen. Therefore, it might be expected that OT would be elevated in the mare at mating. Walmsley (1963) detected a rise in plasma OT after mating in only one of the four mares studied and Alexander et al. (1995) reported an elevation of OT levels in two of the three mares in single samples collected within 5 min after mating. In our study, all five mares showed substantial OT release around the time of mating in agreement with the results of Madill et al. (1998). In two mares, which had to be mounted twice by the stallion, the false mount was associated with release of OT on both occasions. Similarly, in the cow (VanDemark and Hays, 1952) and the ewe (Lightfoot, 1970), mounting without intromission caused an immediate increase in the frequency and amplitude of uterine contractions possibly reflecting OT release.

Intromission and ejaculation are connected with significant tactile stimulation of the vaginal walls, cervix and the uterus. In our study, mechanical stimulation of the external

genitalia and clitoris provoked OT release in two mares. Vaginal distension and cervical manipulation have been reported to release OT in other species (Roberts and Share, 1969; Blank and De Bias, 1977; Schams et al., 1982; Kendrick et al., 1991) as well as in the mare (Betteridge et al., 1985; Aurich et al., 1996; Sharp et al., 1997; Vanderwall et al., 1998). In our study, vaginal distension, cervical stimulation and intrauterine infusion of 500 ml PBS provoked a significant OT response. On several occasions shortly after PBS infusion, the mares adopted a urinating posture or were actually expelling fluid, often coinciding with OT peaks (data not shown). This observation is consistent with the findings of another study, where reflux of infused fluid often occurred within 10 to 15 min post-infusion (Jones, 1995) and it appears in our study that uterine distension provokes uterine contractions, probably elicited by OT release. In our mares, vaginal distension provoked the greatest and most consistent release of OT compared with the other stimuli ($P < 0.05$). Although manipulation of the genital tract caused a significantly greater OT response than mating this is probably due to the longer period of time (12 min) that the stimuli were applied in comparison to the duration of mating in the mare (2 min; McDonnell, 1992). The results of our study demonstrated a gradation in the OT response depending on the duration and severity of the applied stimulus.

Peaks of OT were highest at the time of the stimulus application, and declined rapidly reaching baseline levels by 30–60 min in all experiments. Interestingly, where there was a measurable PGFM response, elevations tended to follow the OT peaks, indicating a possible temporal correlation between the two hormones as already suggested in mares (Vanderwall et al., 1998). No PGFM response was detected at two of the five mating and three of the five teasing episodes. Also, there was no prostaglandin release 16 to 18 h after mating, although increased myoelectrical activity has been reported at that period of time after intrauterine infusion of bacteria to genitally normal mares (Troedsson et al., 1993). However, increased uterine activity could be due to local prostaglandins, released from the inflamed endometrium (Watson, 1989) and detected in uterine flushings (Watson, 1987), which may not reach the circulation in detectable concentrations.

Betteridge et al. (1985) showed in dioestrous mares that vaginal distension, uterine manipulation and cervical dilation increased circulating PGFM levels in 50% of the mares and that rectal palpation and washing of the vulva did not affect PGFM concentrations. In our study, only one oestrous mare had significant PGFM response after manipulation of the genital tract. It has previously been observed that individual mares appear to have different capacities to release $PGF_{2\alpha}$ in response to stimuli applied to the genital tract (Kask et al., 1997). Furthermore, Goff et al. (1987) reported that during oestrus there was a dramatic decline in the PGFM response to exogenous OT possibly as a result of the low endometrial oxytocin receptor levels at this stage of the cycle (Sharp et al., 1997). It also appears that the endometrium may require a period of exposure to progesterone before it has the capacity to release large quantities of $PGF_{2\alpha}$ (Vernon et al., 1981). This may account for failure of $PGF_{2\alpha}$ release in a number of oestrous mares and the low level of release in responding mares in comparison with concentrations measured at luteolysis (Goff et al., 1987).

In conclusion, we have characterised OT release in genitally normal mares around mating and have shown that vaginal distension appears to be a potent stimulus for OT release associated with mating. By comparison, release of $PGF_{2\alpha}$ was observed only in a

minority of mares over all experiments. Future studies should investigate patterns of release of ecbolic hormones after similar stimuli applied to mares with delayed uterine clearance.

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Oxytocin and $PGF_{2\alpha}$ release in mares resistant and susceptible to persistent mating-induced endometritis

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Mares resistant ($n = 7$) and susceptible ($n = 9$) to persistent mating-induced endometritis were artificially inseminated (AI) during oestrus with chilled extended semen. Blood samples were collected from 30 min before AI, for 2 h after AI and again between 16 and 18 h after AI. Samples were assayed for oxytocin and the $PGF_{2\alpha}$ metabolite 13,14-dihydro-15-keto $PGF_{2\alpha}$ (PGFM). AI caused a significant increase in oxytocin concentrations in both the resistant and susceptible mares and there was no significant difference in oxytocin release between the two groups of mares. Mean PGFM concentrations were significantly higher in the resistant group for the first 30 min after AI. In a second experiment, an i.v. injection of oxytocin (1 iu per 20 kg body weight) was administered to both resistant and susceptible oestrous mares. Blood samples were collected at 5 min intervals from 10 min before until 60 min after oxytocin administration. Administration of exogenous oxytocin caused significantly greater $PGF_{2\alpha}$ release in resistant than in susceptible mares. The observed pattern of decreased PGFM concentrations in the susceptible group of mares after both AI and oxytocin administration indicates a possible defect in $PGF_{2\alpha}$ release at the oxytocin receptor or post-receptor level.

Introduction

Introduction of bacteria and spermatozoa into the uterus at the time of mating in mares can cause a transient inflammation of the endometrium (Kotilainen *et al.*, 1994). Uterine contractility promotes uterine clearance, eliminating cellular debris and uterine fluid (LeBlanc *et al.*, 1994), presumably by drainage via the cervix and lymphatic system. It has been suggested that mares failing to evacuate uterine fluid and cellular debris within 12–48 h (LeBlanc *et al.*, 1994; Katila, 1995) from the time of uterine challenge have impaired uterine contractility (Troedsson and Liu, 1991). These mares are classified as having persistent mating-induced endometritis (PMIE). Mares susceptible to PMIE have reduced myoelectrical activity after the introduction of infection into the uterus compared with resistant mares (Troedsson *et al.*, 1993a).

Artificial insemination (AI) is used widely as a management tool to minimize uterine contamination in mares susceptible to endometritis (Kenney *et al.*, 1975). In other species, the release of oxytocic hormones at the time of breeding (Fox and Knaggs, 1969; McNeilly and Ducker, 1972; Fuchs *et al.*, 1981; Schams *et al.*, 1982; Todd and Lightman, 1986; Claus and Schams, 1990) directly influences uterine contractility and possibly assists with gamete transport (McNeilly and Folley, 1970; Wathes, 1984). Oxytocin can affect uterine motility both directly and indirectly via release of $PGF_{2\alpha}$ (Poyser, 1995). The responsiveness of the uterus to oxytocin and production and release of $PGF_{2\alpha}$ is modulated in mares via uterine oxytocin receptors (Sharp *et al.*, 1997). It is possible that

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oxytocin and $PGF_{2\alpha}$ released at the time of AI in mares could promote uterine clearance and that mares with PMIE could have deficient oxytocic hormone release.

The aims of the present study were: (i) to investigate whether there are any differences in the release of oxytocin and $PGF_{2\alpha}$ before, during, and after AI with chilled extended semen in mares resistant and susceptible to PMIE; and (ii) to determine the responsiveness of the uteri of these mares to exogenous oxytocin.

Materials and Methods

Animals

Fertile mares ($n = 11$), aged 5–15 years, body weight 340–520 kg, classed as genitally normal by their reproductive histories and endometrial biopsy scores of 1–2A (Kenney and Doig, 1986), were used. The susceptible group of mares consisted of 13 mares, aged 7–18 years, body weight 370–620 kg, that were referred to our reproduction clinic with a history of subfertility related to accumulation of uterine fluid containing many neutrophils for at least 48 h after mating. Only mares with no obvious conformational vulvar and perineal problems or cervical defects were included in the study. Oestrus was detected by teasing with a stallion and transrectal ultrasonography of the genital tract. Mares were considered to be in oestrus if they responded positively to teasing, uterine oedema was present and a follicle ≥ 35 mm in diameter was present in the ovaries.

Experiment 1

Resistant ($n = 7$) and susceptible ($n = 9$) mares were used. On the day each mare was detected as being in oestrus, an indwelling cannula (13 gauge; Presidio Medico, Ecouen, France) was placed in the jugular vein under local anaesthesia after surgical preparation of the area. Blood samples for oxytocin and 13,14-dihydro-15-keto $PGF_{2\alpha}$ (PGFM), the main $PGF_{2\alpha}$ blood plasma metabolite (Goff *et al.*, 1984), were collected for 30 min at 2 min intervals, after which time the mares were artificially inseminated using the hygienic procedures described by Watson (1995). Blood sampling continued at 2 min intervals during AI and for 1 h after AI, and for a further 1 h at 5 min intervals. Additional samples for PGFM analysis were collected at 15 min intervals between 16 and 18 h after AI.

Experiment 2

Resistant ($n = 11$) and susceptible ($n = 10$) oestrous mares were used. An indwelling cannula (13 gauge; Presidio Medico) was placed in the jugular vein under local anaesthesia after surgical preparation of the area and blood samples were obtained at 5 min intervals from 10 min before until 60 min after i.v. oxytocin administration (1 iu per 20 kg body weight; Oxytocin-S; Intervet, Cambridge).

Sample handling

Blood samples were collected into evacuated heparinized tubes and immediately placed on ice until separation. The samples were centrifuged at 2000 *g* for 15 min at 4°C. Each plasma aliquot was acidified with 10 mmol acetic acid l^{-1} (10 µl per ml plasma) for oxytocin assay. Mild acidification of blood plasma improves oxytocin recovery rates in goats (Homeida and Cooke, 1984). All samples were frozen initially at –70°C and stored subsequently at –20°C until assayed.

Semen sampling

Semen was collected from a fertile stallion aged 5 years. The same stallion was used for all inseminations. On the day of collection, the gel was separated by filtration and the semen was diluted with Kenney's skimmed milk semen extender (containing 1 mg ticarcillin ml^{-1} (Beecham

Research Laboratories, Brentford)) to a concentration of 25×10^6 progressively motile spermatozoa per ml in a total volume of 40 ml. The extended semen was chilled overnight in an insulated container (Equitainer; Hamilton-Thorn, Beverley, MA). Mixed bacterial growth was observed by cultural examination of undiluted semen. After 48 h no growth was observed in either extended semen incubated overnight at 37°C or extended semen chilled overnight.

Oxytocin assay

Oxytocin was extracted from plasma using C_{18} SepPak cartridges (Waters Chromatography, Milford, MA) and the radioimmunoassay was carried out as described by Thornton *et al.* (1986) using an antiserum described by Sheldrick and Flint (1981). The extraction recovery rate was 74.8%. The detection limit for the assay was 0.8 pg oxytocin ml^{-1}. The intra- and inter-assay coefficients of variation were 6.6 and 11.7%, respectively.

PGFM assay

Concentrations of the main initial $PGF_{2\alpha}$ plasma metabolite PGFM were measured to monitor $PGF_{2\alpha}$ release (Granstrom and Kindahl, 1982). The assay was performed with unextracted plasma. Before the addition of antibody and radioactive tracer, 0.3 ml 0.25% (w/v) bovine gamma globulin (Sigma Chemical Co, Poole) in buffer was added and the tubes were heat-treated for 30 min at 45°C. The antibody crossreacted with 15-keto-$PGF_{2\alpha}$ (16.0%), 13,14-dihydro-$PGF_{2\alpha}$ (4.0%) and 15-ketodihydro-PGE_2 (1.7%). The degree of crossreactivity with all other prostaglandins tested was < 0.1%. The detection limit of the assay was 20 pg ml^{-1}. The intra- and inter-assay coefficients of variation were 6.6 and 11.7%, respectively. Concentrations less than the detection limit of the assay were taken as equal to the detection limit of the assay.

Statistical analysis

Baseline hormone concentrations were calculated from the mean of the values obtained before AI. The response to AI for both hormones was obtained from the mean values of the samples corresponding to the concentrations of the peak immediately after the application of the stimulus until hormone concentrations returned to baseline values (Table 1). Mean hormone concentrations were calculated for each 30 min interval thereafter. The mean concentrations of both hormones at all time intervals and the magnitude of the response to AI were compared between the two groups using two-sample *t* tests.

Responses of individual mares were also assessed because of the pulsatile nature and short half-life of oxytocin release. Mares were considered to have a positive oxytocin response if the mean concentration of the peak immediately after AI was greater than the mean baseline concentration + 2 SD. Mean PGFM concentrations were calculated for each mare as above (30 min intervals) and a positive response was recorded if the increase for each mare was greater than the mean baseline concentration + 2 × intra-assay coefficient of variation in the first 30 min after AI. The number of mares from each group responding to AI with hormone release was compared using Fisher's exact test.

In Expt 2, PGFM concentrations for both groups of mares were compared using a two-sample *t* test for each 5 min interval.

Results

Experiment 1

Baseline oxytocin concentrations were not significantly different between the resistant and susceptible groups (Table 1). There was a significant increase in oxytocin concentration in response

Table 1. Oxytocin and 13,14-dihydro-15-keto $PGF_{2\alpha}$ concentrations for each 30 min interval before and after artificial insemination in mares resistant ($n = 7$) and susceptible ($n = 9$) to persistent mating-induced endometritis

	Resistant		Susceptible	
Time	Oxytocin (pg ml^{-1})	PGFM (pg ml^{-1})	Oxytocin (pg ml^{-1})	PGFM (pg ml^{-1})
30 min before AI	5.7 ± 1.1	22.7 ± 0.7	6.3 ± 1.9	–
at AI	15.1 ± 3.2	27.4 ± 4.2	10.9 ± 2.1	–
AI to 30 min after AI	7.6 ± 2.3	34.1 ± 6.0*	8.5 ± 2.2	–
30–60 min after AI	9.6 ± 2.8	80.8 ± 54.5	8.4 ± 1.9	–
60–90 min after AI	8.4 ± 2.3	139.2 ± 95.8	6.7 ± 1.7	–
16–18 h after AI	nd	32.6 ± 11.5	nd	45.2 ± 18

PGFM: 13,14-dihydro-15-keto $PGF_{2\alpha}$. AI: artificial insemination. nd: not determined. –: below detection limit of assay (taken as equivalent to the detection limit of the assay (20 pg ml^{-1}) for statistical analysis).
*Values are significantly different between resistant and susceptible mares ($P < 0.05$).
Values are mean ± SEM.

to AI ($P < 0.05$) in both groups compared with baseline concentrations (Table 1). The mean interval from the start of AI until oxytocin concentrations returned to baseline was 7.24 ± 0.92 min. There was no difference between the two groups in the magnitude of the oxytocin response to AI and the mean oxytocin concentration for any of the 30 min periods after AI (Fig. 1a). There was also no significant difference between the number of resistant ($n = 5$; 71%) and susceptible ($n = 3$; 33%) mares releasing oxytocin in response to AI.

Baseline PGFM concentrations were below the detection limit of the assay in all the susceptible mares (Table 1). PGFM concentrations did not increase at the time of AI in either group of mares, but mean PGFM concentrations for the first 30 min after AI were significantly higher ($P < 0.05$) in the resistant group (Table 1) than in the susceptible group. Furthermore, significantly more ($P < 0.05$) resistant ($n = 5$; 71%) than susceptible ($n = 0$) mares released $PGF_{2\alpha}$ in response to AI (Fig. 1b). There was no significant difference between the resistant and susceptible groups of mares in either oxytocin or PGFM concentration during any other 30 min period, despite the very high PGFM concentrations observed in some of the resistant mares. There was no difference in mean PGFM concentrations 16 to 18 h after AI between the two groups of mares and concentrations were not significantly higher than baseline samples.

Experiment 2

There was no difference in mean baseline PGFM concentrations between the two groups of mares before oxytocin administration (Fig. 2). However, the proportion of mares that released $PGF_{2\alpha}$ in response to oxytocin in the resistant group ($n = 10$; 91%) was significantly higher ($P < 0.05$) than in the susceptible group ($n = 4$; 40%). The PGFM concentrations in the resistant group were significantly higher ($P < 0.05$) than the concentrations in the susceptible group at all times after oxytocin administration until the end of the experiment (Fig. 2). PGFM concentrations in the responding susceptible mares returned to baseline concentrations within 20 min but had not returned to baseline concentrations in the resistant group by the end of the experiment (after 60 min).

Discussion

The oxytocic hormones, oxytocin and $PGF_{2\alpha}$, regulate myometrial contractility in many species (women: Seitchik and Chatkoff, 1976; pigs: Garcia-Villar *et al.*, 1983; Carnahan *et al.*, 1996; dogs:

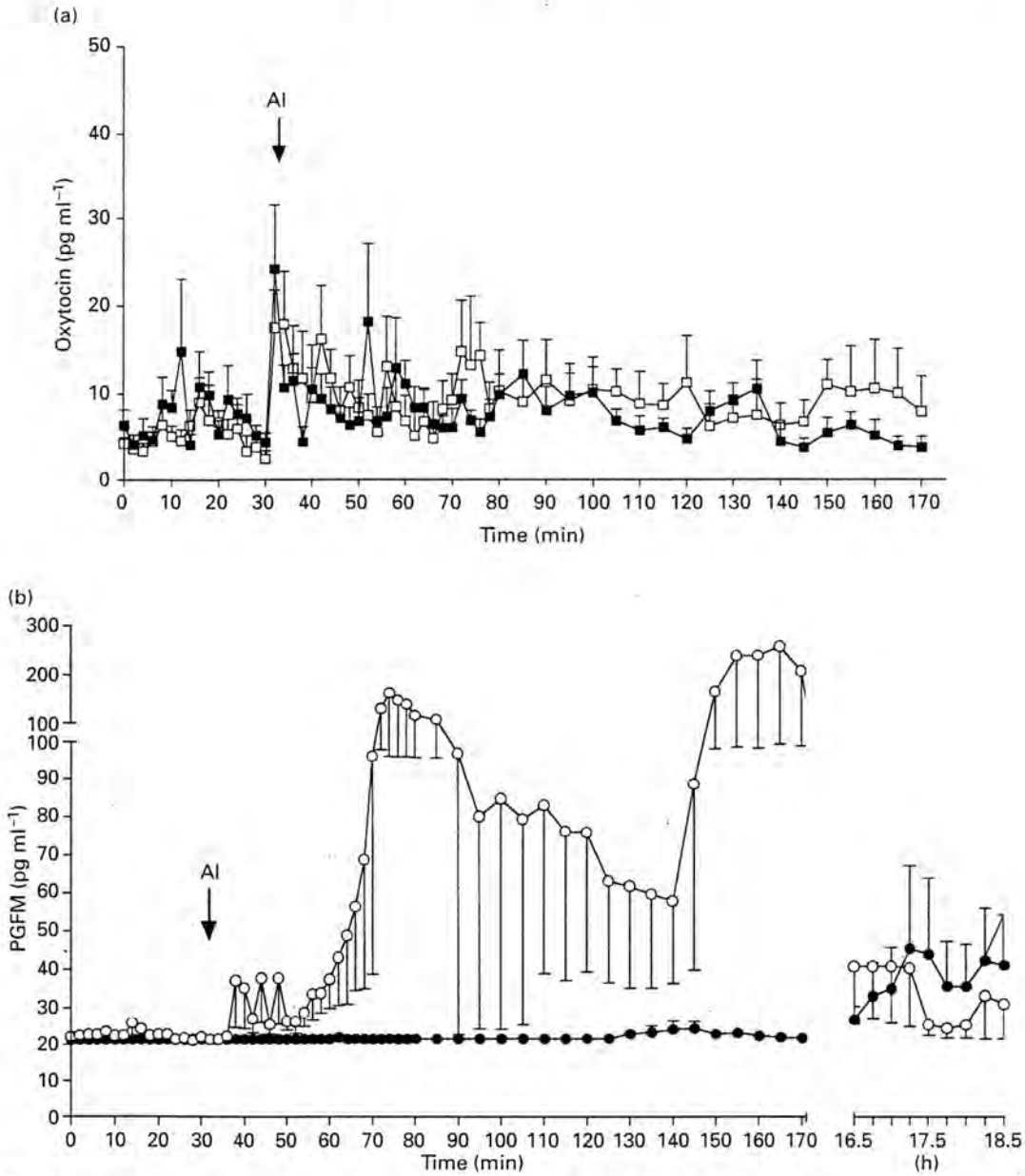

Fig. 1. Mean ± SEM (a) oxytocin and (b) 13,14-dihydro-15-keto $PGF_{2\alpha}$ (PGFM) concentrations around the time of artificial insemination (AI) in mares resistant (□ or ○; $n = 7$) and susceptible (■ or ●; $n = 9$) to persistent mating-induced endometritis.

Wheaton and Barbee, 1993), including mares (Goddard and Allen, 1985; Cross and Ginther, 1987; Ko *et al.*, 1989). Mares susceptible to endometritis have delayed uterine clearance and it has been suggested that such mares have impaired uterine contractility (Evans *et al.*, 1986; Troedsson and Liu, 1991; LeBlanc *et al.*, 1994). This is thought to be the first study that compares oxytocic hormone release profiles at the time of mating in mares resistant and susceptible to endometritis.

Patterns of oxytocin release have been reported in many species. However, reports for mares are

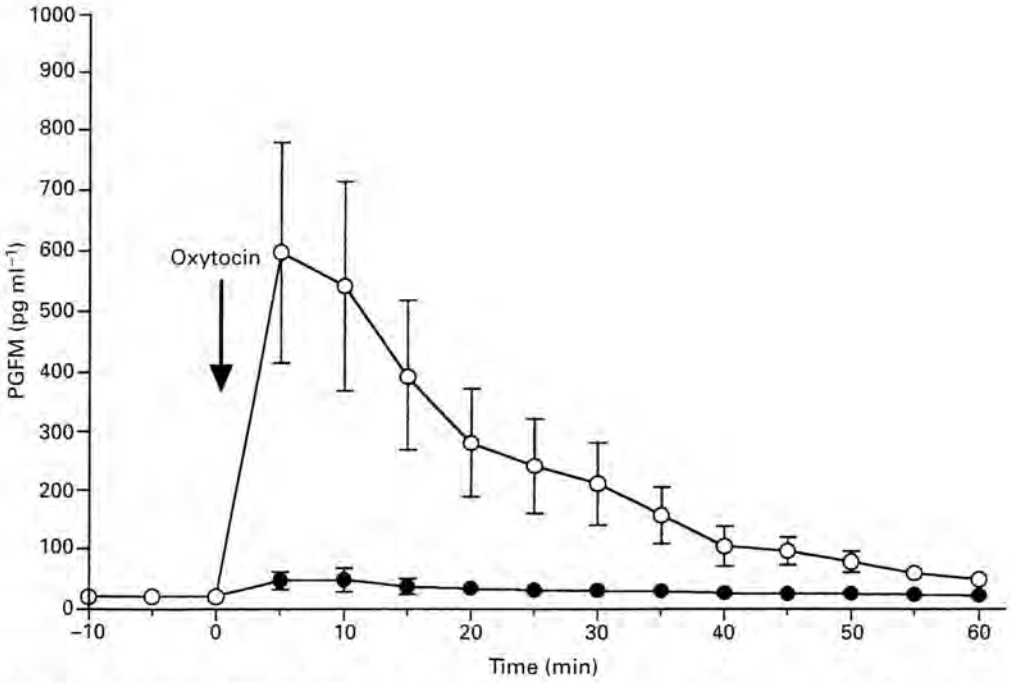

Fig. 2. Mean ± SEM 13,14-dihydro-15-keto $PGF_{2\alpha}$ (PGFM) concentrations after i.v. oxytocin administration (1 iu per 20 kg body weight; Oxytocin-S; Intervet, Cambridge) to oestrous mares resistant (○; $n = 11$) and susceptible (●; $n = 10$) to persistent mating-induced endometritis.

contradictory and inconclusive (Burns *et al.*, 1981; Tetzke *et al.*, 1987; Stevenson *et al.*, 1991; Alexander *et al.*, 1995). In contrast to ruminants (for review see Wathes, 1989), oxytocin is released only from the posterior pituitary gland in mares and not from the ovaries (Stevenson *et al.*, 1991), although a contribution from the uterus cannot be ruled out (Behrendt *et al.*, 1997; Watson *et al.*, 1997). Oxytocin binds to uterine receptors located in the endometrium and myometrium of mares (Stull and Evans, 1986; Sharp *et al.*, 1994), and provokes uterine contractions by increasing the influx of calcium into myometrial cells (Csapo, 1962). However, oxytocin also stimulates uterine contractility indirectly by mobilising arachidonic acid and initiating the production of uterine $PGF_{2\alpha}$ (for review see Poyser, 1995).

In the present study, it is demonstrated that AI can stimulate the release of oxytocin in mares, which supports the observations of Alexander *et al.* (1995). This is probably due to release resulting from distension of the vaginal walls (Aurich *et al.*, 1996) and manipulation of the cervix (Sharp *et al.*, 1994) at the time of semen deposition into the uterus. However, other factors might play a role in the release of oxytocin, including distension of the uterine walls by the inseminate and the antigenic challenge of the spermatozoa (E. Nikolakopoulos and E. D. Watson, unpublished). In the present study, oxytocin concentrations before and after AI were similar in both resistant and susceptible mares. Troedsson *et al.* (1993a) reported an initial increase in myoelectrical activity in both resistant and susceptible mares for the first 10–15 min after infusion of bacteria into the uterus, which correlates well with the pattern of oxytocin release observed after AI in the present study. However, there is no information on the relationship between plasma concentrations of oxytocin and myometrial activity in mares. In one study of ewes it was demonstrated that minute doses of oxytocin similar to endogenous pulses stimulate uterine motility (Gilbert *et al.*, 1991), but this finding was not substantiated in another study (Garcia-Villar *et al.*, 1983). In mares, the low oxytocin

receptor content of the uterus at oestrus (Sharp *et al.*, 1997) does not correlate with the increased contractile response measured at this stage of the cycle (Jones *et al.*, 1991).

In the present study, mean PGFM concentrations were increased significantly for the first 60 min after AI in the genitally normal mares. Other studies have demonstrated similarly that $PGF_{2\alpha}$ is released after manipulation of the tract at embryo transfer and after uterine lavage (Betteridge *et al.*, 1985; Watson *et al.*, 1988; Kask *et al.*, 1997). In the present study, the oxytocin peak after AI may have stimulated $PGF_{2\alpha}$ release, indicating that there may be a temporal relationship between the two hormones in mares, as suggested by Sharp *et al.* (1994) and Vanderwall *et al.* (1998). However, an important finding in the present study was that no detectable concentrations of PGFM were measured in susceptible mares, despite the similarity of oxytocin profiles between the two groups. There is little information on the relative importance of oxytocin and prostaglandin in the regulation of uterine contractions, although it is thought that both are involved in women (Fuchs, 1987) and rats (Maggi *et al.*, 1991). In reproductively normal mares, treatment with phenylbutazone, an inhibitor of prostaglandin synthesis at oestrus, substantially interferes with uterine clearance (Cadario *et al.*, 1995), indicating clearly that endogenous prostaglandin is important in myometrial contractility. Some mares classified as resistant in the present study only released low amounts of $PGF_{2\alpha}$ at AI. The variability in oxytocic hormone response among mares and clinical observations (E. D. Watson, unpublished) indicate that resistance and susceptibility to PMIE may include a spectrum of intermediate categories. However, many other documented factors contribute to susceptibility to PMIE, including external vulvar and perineal conformation (Pascoe, 1979) and lymphatic clearance (LeBlanc *et al.*, 1995). The diversity in the hormonal results obtained in the present study may reflect a lack of uniformity among the clinical cases investigated. It is accepted widely that mares should be classified as resistant or susceptible to PMIE based on inability to clear bacteria after intrauterine bacterial challenge (Troedsson *et al.*, 1993b). However, classification of mares has been refined recently, and it has been demonstrated that some mares with delayed uterine clearance eliminate intrauterine bacteria after challenge (Nikolakopoulos and Watson, 1999). Furthermore, it is not possible to experimentally infect client-owned mares with bacteria. On the basis of the current understanding of PMIE, the selected clinic cases were considered to be representative of susceptible mares.

Susceptible mares are described as having an approximately 2 h delay in the onset of myometrial activity after intrauterine bacterial infusion and low myoelectrical activity from 11 h after infection compared with the high activity observed in resistant mares (Troedsson *et al.*, 1993a). In the interval immediately after AI, prostaglandin release was low in susceptible mares, which might explain the delay in myometrial activity reported by Troedsson *et al.* (1993a). However, in the present study no differences were observed in the PGFM concentrations between resistant and susceptible mares 16–18 h after AI. Troedsson *et al.* (1993a) suggested that the high myometrial activity observed in resistant mares is caused by prostaglandins released from activated inflammatory cells. It is possible that the high prostaglandin concentrations produced by the inflamed endometrium (Watson, 1989) act locally and, thus, cannot be measured in the blood. However, in contrast to this, Watson *et al.* (1987) observed that increased PGFM concentrations in mares with acute endometritis correlated well with uterine luminal $PGF_{2\alpha}$ concentrations. Therefore, in the present study, there were no apparent differences in the circulating prostaglandin concentrations at 16–18 h after AI that could account for the observed differences in myometrial activity.

The significantly lower $PGF_{2\alpha}$ release observed in the susceptible group of mares at the time of AI, despite the release of oxytocin, indicates that there is a defect in $PGF_{2\alpha}$ production. Oxytocin administration is used as an *in vivo* stimulus for $PGF_{2\alpha}$ release in many species (Beard *et al.*, 1994; Mann and Lamming, 1995; Carnahan *et al.*, 1996), including mares (Goff *et al.*, 1987), and acts via the oxytocin receptor (Sharp *et al.*, 1997). In Expt 2, the significant response of the resistant mares in oestrus to oxytocin was in marked contrast to the observations of Goff *et al.* (1987), who observed an abrupt decrease in the $PGF_{2\alpha}$ response of oestrous mares in response to oxytocin administration. These differences may be due to the higher oxytocin dose used in the present study. The ability to release prostaglandin in response to oxytocin may explain the high contractile activity of the uterus at oestrus. More importantly, in the present study it was demonstrated that most susceptible mares

failed to respond to oxytocin and those that did had a significantly lower response than the resistant group. This result may explain the reduced prostaglandin response of susceptible mares at AI and the reduced contractility of the uterus.

Administration of oxytocin to mares after mating is used in most of the treatment protocols for PMIE (Allen, 1991; LeBlanc, 1994; Pycock and Newcombe, 1996) to eliminate accumulated intrauterine fluid by provoking powerful contractions visible by transrectal ultrasonography (Nikolakopoulos and Watson, 1997). However, although oxytocin administration affects uterine activity in susceptible mares (LeBlanc *et al.*, 1994; Nikolakopoulos and Watson, 1997) and can eliminate small volumes of fluid after inhibition of $PGF_{2\alpha}$ synthesis in normal mares (Cadario *et al.*, 1995), some clinically affected mares fail to respond to oxytocin therapy (E. D. Watson, unpublished). Some of the mares included in the present study that failed totally to release $PGF_{2\alpha}$ at AI continued to accumulate fluid and failed to become pregnant, despite intensive flushing and oxytocin therapy. Treatment with $PGF_{2\alpha}$, which has a more sustained effect than oxytocin (Troedsson *et al.*, 1995; Combs *et al.*, 1996), might be a more appropriate form of therapy for such mares. However, the variability of prostaglandin responses after AI and oxytocin administration among resistant and susceptible mares indicates further that separation of mares into only two distinct categories may not always be appropriate.

In conclusion, oxytocin release profiles do not differ, before, during and after AI between resistant and susceptible mares. However, significantly fewer susceptible mares than resistant mares released $PGF_{2\alpha}$ in response to endogenous or exogenous oxytocin administration, indicating that there is a defect at the receptor or post-receptor level. The variability in oxytocic hormone profiles indicates that there may be a gradation in susceptibility and resistance to PMIE in mares.

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Organisation of uterine innervation in the mare: distribution of immunoreactivities for the general neuronal markers protein gene product 9.5 and PAN-N

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Introduction

It has been well documented that the mammalian female reproductive tract receives an extensive nerve supply from different populations of nerves that can be visualised by specific immunohistochemical techniques. The nerve supply comprises not only autonomic nerves including adrenergic and cholinergic, but also peptidergic nerves (Papka *et al.* 1985; Wrobel and Kujat 1993). However, the density and the populations of these nerves seem to vary between species (Huang *et al.* 1984; Renegar and Rexroad 1990; Majewski *et al.* 1995). Previous studies have demonstrated that uterine innervation is influenced by a large number of mechanical, physiological and hormonal factors. The uterine nerve supply decreases significantly during pregnancy (Alm *et al.* 1986; Lundberg *et al.* 1988; Moustafa 1988) and also changes throughout the oestrous cycle (Zoubina *et al.* 1998).

Protein gene product 9.5 (PGP) is a general marker for neurons, having a molecular weight of about 27 kDa (Thompson *et al.* 1983). It has been used widely as a pan-neuronal marker to visualise the entire uterine innervation in the rat (Zoubina *et al.* 1998) and guinea pig (Alm *et al.* 1988; Lundberg *et al.* 1988). The neurofilament marker PAN-N is composed of 3 antibodies that react with the low, medium and high molecular weight neuronal filament proteins (NF-L [68–70 kDa], NF-M [150 kDa] and NF-H [200–210 kDa]). Consequently, it is a convenient marker for neuronal cells and neuronal processes in general, and allows differentiation from non-neuronal cells. Zoubina *et al.* (1998) have shown that PGP-immunoreactive nerves are found in greatest abundance in the vascular zone of the myometrium, which is the area of blood vessels and connective tissue that is interposed between the outer longitudinal and inner circular smooth muscle layers of the uterus. The circular and longitudinal smooth muscle layers themselves contain moderate numbers of nerve fibres, while innervation of the endometrium is significantly lower than in other areas.

Uterine motility is known to play a role in fertility in the mare (Troedsson *et al.* 1993; LeBlanc *et al.* 1994) and is controlled by a combination of hormonal and neuronal mechanisms. There is no published information on the organisation of uterine innervation in the mare. Therefore, the present study was undertaken to investigate uterine innervation in the mare using primary antisera against PGP and PAN-N.

Materials and methods

Tissue samples were obtained *postmortem* from 11 mares age 3–18 years. The reproductive tract was removed within 20 min of euthanasia, was washed gently and then dissected in 0.1 mol/l phosphate-buffered saline (PBS). Four to 6 segments were collected from each of the following regions: uterine horn, body and cervix. Samples were then fixed immediately in cold 4% paraformaldehyde (pH 7.2) for 14–16 h at 4°C. Samples were transferred into 0.1 mol/l PBS (pH 7.2) containing 30% sucrose and stored for 24 h at 4°C. Tissues were snap frozen in optimum cutting temperature (OCT) compound[1] in an isopentane/dry-ice slurry, then stored at -70°C.

Immunohistochemical procedure

Cryostat sections (12–15 µm) were cut and processed for immunohistochemistry using the avidin-biotin complex method[2] described previously (Watson and Thompson 1996). Briefly, sections were air-dried for 60–90 min at room temperature, rinsed in 0.01 mol/l PBS (pH 7.2) and blocked with 3% H_2O_2 in methanol for 30 min to reduce nonspecific binding. Slides were then preincubated with 1% goat antiserum in 0.01 mol/l PBS for 1 h. Primary antibodies[3] raised in rabbits were used at a dilution of 1:8000 for both PGP and PAN-N. The specificity of these antibodies had been characterised (Thompson *et al.* 1983; Wilson *et al.* 1988). The sections were incubated with primary antibodies overnight at 4°C, then washed 3 times in PBS and incubated with the second antibody[2] (goat anti-rabbit) diluted in PBS containing 1% normal goat serum for 2 h at room temperature. The sections were washed thoroughly in PBS, then incubated with the avidin-biotin complex for a further 30 min and rinsed 3 further times with PBS. Chromagen[2] (3-amino-9-ethylcarbazole: AEC) was added as the final substrate (red reaction product) and incubated at room temperature in the dark for 10–15 min. Each slide was then rinsed carefully with distilled water. The sections were normally counterstained with Meyer's haematoxylin; however, sometimes sections with fine nerve fibres were excluded from this step. As negative controls, sections were included in which the first antibody was replaced by normal rabbit serum. Sections of equine jejunum and feline uterus were included on each slide to act as positive controls. The sections were examined by microscopy and the relative density of the immunoreactive nerves

Fig 1: Protein gene peptide 9.5-immunoreactive nerve bundles and fibres in the myometrium of the uterine body (arrows). Positive staining is counterstained dark with haematoxylin (x20).

Fig 2: PAN-N-immunoreactive nerve bundles in the cervix (arrows). Positive staining is dark and counterstaining was excluded to show delicate neurofilaments in the nerve bundles (x20).

graded semiquantitatively as: - = not found; ± = very few; + = a small number of fibres; ++ = a moderate number of fibres and +++ = a large number of fibres.

Results

Density and distribution of immunostaining for both PGP and PAN-N were consistent for each site examined in all mares. Within the uterus, staining density for both PGP-IR and PAN-N-IR was greatest in the myometrial smooth muscle layers and the intramyometrial vascular zone, and less in the endometrium. There were no marked differences between the supply to uterine horn or body, but nerve density was slightly greater in the cervix (Table 1). PGP-IR (Fig 1) and PAN-N-IR (Fig 2) nerve bundles and fine nerve fibres were often oriented in parallel to uterine smooth muscle bundles in the inner and outer smooth muscle layers and frequently ran close to blood vessels. In the endometrium, PGP-IR and PAN-N-IR nerves were present mostly as fine nerve fibres in the luminal epithelium and around endometrial glands. In general, the pattern of expression of the PGP-IR and PAN-N-IR neurofilaments was similar; however, more nerve bundles were positive for PAN-N-IR, and immunostaining for PGP identified more delicate nerve fibres in the endometrium and near blood vessels. For both primary antibodies, the positive control sections showed clear positive staining of immunoreactive nerves and negative control sections failed to demonstrate any positive staining.

Discussion

In this study, we have described the general uterine innervation in the mare for the first time and shown that, as in other species, the equine uterus receives an abundant nerve supply. Two different general neuronal markers were used, because PGP can pick up small nerve fibres better than PAN-N while PAN-N is more likely to miss small diameter fibres than large fibres. In this study, nerve bundles were more immunoreactive for PAN-N, whereas nerve fibres tended to be positive for PGP-IR.

A variety of immunoreactive nerve bundles and fine nerve fibres were present in the uterus and cervix. The outer myometrial smooth muscle layers contained predominantly nerve bundles, while inner myometrial smooth muscle layers, the intramyometrial vascular zone and the endometrium contained more fine nerve fibres. This is in agreement with previous studies in the rat (Zoubina *et al.* 1998) and guinea pig (Alm *et al.* 1988; Lunberg *et al.* 1988). In our study, the density of uterine innervation was greatest in the myometrial smooth muscle layers and in the intramyometrial vascular zone, and was least in the endometrium. Our results were in agreement with other reports that the density of innervation was greater in the cervix than in the uterus (Zoubina *et al.* 1998). The large number of nerve fibres in the cervix may have a role in regulating cervical tone in dioestrus, during pregnancy and at parturition in the mare.

Contractility of the uterus is regulated by the interaction and coordination of myogenic, neurogenic and hormonal factors. Dysfunction of uterine contractility in the mare arising from problems with neuronal control mechanisms could potentially lead to reproductive problems, including delay in clearance of intrauterine fluid after mating and problems during parturition. As it is known that persistent mating-induced endometritis (PMIE) is the most common cause of subfertility in broodmares, it is important to understand the role of neuronal control of uterine motility in the equine reproductive system. In conclusion, we have shown that the uterus of the mare receives an extensive nerve supply that probably plays an important role in controlling its reproductive functions, including uterine

TABLE 1: Distribution and relative frequency of PGP- and PAN-N-IR nerves in equine uterine horn, body and cervix

Primary antibody	Uterine horn		Uterine body		Cervix
	Endometrium	Myometrium	Endometrium	Myometrium	
PGP 9.5	+	++	+	++	+++
PAN-N	+	++	+	++	+++

Semiquantitative evaluation of the density of the immunoreactive nerves: - = not found, ± = very few, + = small number of fibres, ++ = moderate number of fibres and +++ = large number of fibres.

myometrial contractility, as well as lymphatic drainage. Future studies should investigate uterine innervation in mares displaying defects in myometrial contractility, such as mares with persistent mating-induced endometritis.

Manufacturers' addresses

[1]Miles Inc., Elkhart, Indiana, USA.
[2]Vector Laboratories, Peterborough, Cambridgeshire, UK.
[3]Affinity Research Products, Exeter, Devon, UK.

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Research

Immunohistochemical study of the distribution of adrenergic and peptidergic innervation in the equine uterus and the cervix

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Little is known about neurogenic regulation of uterine contractility in mares. The present study investigated the distribution of adrenergic and peptidergic nerves in the mare uterus. Samples from the uterine horn, body and cervix were collected from 18 cyclic mares for immunohistochemistry. The uterus was well supplied with adrenergic nerves. A large number of tyrosine hydroxylase- and dopamine β-hydroxylase-immunoreactive nerve bundles and fibres were present in the myometrium and endometrium in all regions of the uterus and cervix. These adrenergic nerve bundles and fibres travelled parallel to the muscle layers and were often associated with blood vessels. The density of peptidergic nerves was less than that of adrenergic nerves, but the pattern of distribution was similar. Neuropeptide Y-immunoreactive nerve fibres were the most abundant, whereas vasoactive intestinal polypeptide- and calcitonin gene-related peptide immunoreactive nerve fibres were less frequently seen. Substance P-immunoreactive nerve fibres were the most sparse. Peptidergic nerves were distributed among the smooth muscle layers and near endometrial glands and were often associated with blood vessels in all regions of the uterus. The density of peptidergic nerve fibres was similar in the uterine horn and body but was slightly denser in the cervix. These findings indicate that uterine innervation may have an important role in controlling reproductive functions in mares.

Introduction

Uterine contractility is regulated by the coordination and interaction of myogenic, neurogenic and hormonal control mechanisms. The main external factors that control uterine contractility are the sex steroid hormones, oestrogen and progesterone, and also oxytocin and prostaglandins. In mares, uterine contractility is of key importance in uterine clearance after mating (Troedsson *et al.*, 1993). Mares susceptible to endometritis induced by persistent mating may have impaired uterine contractile activity (Troedsson *et al.*, 1993; Nikolakopoulos and Watson, 1997) and defects in innervation or nerve function may contribute to this condition. However, there have been no detailed studies on innervation of the equine uterus.

Previous studies on innervation of the mammalian female reproductive tract have demonstrated that it is well supplied by the autonomic nervous system, with adrenergic nerve fibres predominating (Thorbert *et al.*, 1977; Alm and Lundberg, 1988; Mitchell and Ahmed, 1992). Tyrosine hydroxylase (TH) and dopamine β-hydroxylase (DβH) are catecholamine-synthesizing enzymes and their presence has been used to demonstrate the adrenergic contribution to uterine innervation (Alm and Lundberg, 1988; Wrobel and Kujat, 1993). In contrast to other organs, uterine adrenergic innervation is affected by ovarian steroid hormones during the oestrous cycle and pregnancy (Marshall, 1981; Zoubina *et al.*, 1998). Adrenergic innervation is important in the neuronal control of uterine blood flow, myometrial contractility, and endometrial secretory function (Heinrich *et al.*, 1986) and exerts biological effects consistent with a regulatory function in female reproduction (Taneike *et al.*, 1991).

In addition to adrenergic and cholinergic nerves, the mammalian reproductive tract is also supplied by peptidergic nerves containing a variety of neuropeptides. These peptidergic nerves vary in type and density among species (Huang *et al.*, 1984; Heinrich *et al.*, 1986). Various neuropeptides are present or coexist in nerve fibres of the female reproductive tract, and these nerves are thought to be involved in the control of reproductive organs. Neuropeptide Y (NPY), vasoactive intestinal polypeptide (VIP), calcitonin gene-related peptide (CGRP), and substance P (SP) are thought to play important roles in the control of myometrial contractility (Mukai *et al.*, 1991) and uterine blood flow (Ekesbo *et al.*, 1991). As these nerves are particularly abundant in the lower genital tract, they may also play a role in regulation of uterine activity during pregnancy and parturition (Majewski *et al.*, 1995; Houdeau *et al.*, 1997).

In the present study adrenergic innervation of the uteri of genitally normal mares was investigated using the catecholamine enzymatic markers TH and DβH. The distribution of nerve fibres immunoreactive for the peptide neurotransmitters NPY, CGRP, VIP and SP was also investigated.

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Materials and Methods

Animals

Uterine tissue samples were collected from 18 cyclic mares (aged between 3 and 16 years and weighing 390–520 kg) killed for reasons other than reproductive problems. The stage of the ovarian cycle was established by determining peripheral blood progesterone concentrations as well as by the presence or absence of a corpus luteum in the ovaries, the presence of a follicle > 30 mm in diameter and uterine oedema. The reproductive tracts of these mares (seven oestrous mares and 11 dioestrous mares) were removed within 20 min of death and were gently washed and dissected in 0.1 mol PBS l^{-1} (pH 7.2). Samples were collected from the uterine horn ($n = 4$), body ($n = 4$) and cervix ($n = 4$) from each mare. Samples were then fixed immediately in 4% (w/v) paraformaldehyde (pH 7.2) for 14–16 h at 4°C. Samples were transferred into 0.1 mol PBS l^{-1} (pH 7.2) containing 30% (v/v) sucrose and stored in this solution for at least 24 h at 4°C, with four changes of buffer. Tissues were snap frozen in OCT compound (Miles Inc., Elkhart, IN) in an isopentane–dry ice slurry and then stored at –70°C before sectioning.

Immunohistochemical procedure

Cryostat sections 12–15 μm in thickness were cut and mounted on slides coated with Bio-Bond (British BioCell International, Cardiff). Sections were air-dried for 60–90 min at room temperature, rinsed in 0.01 mol PBS l^{-1} (pH 7.2) for 10 min and blocked with 3% H_2O_2 in methanol for 30 min to reduce non-specific binding. Slides were then preincubated with 1% goat antiserum in 0.01 mol PBS l^{-1} for 1 h. The excess serum was blotted and the sections were incubated with primary antibody at the appropriate dilution overnight at 4°C before washing three times in 0.01 mol PBS l^{-1}. Primary antisera were raised in rabbits, and were used at the following dilutions in PBS: TH, 1:6000 (Affiniti Research Products, Exeter); DβH, 1:4000 (Affiniti Research Products); NPY, 1:4000 (Affiniti Research Products); VIP, 1:4000 (Peninsula Laboratories Europe, St Helens); CGRP, 1:4000 (Peninsula Laboratories Europe); and SP, 1:6000 (Peninsula Laboratories Europe). The sections were incubated with the second antibody (goat anti-rabbit) in PBS containing 1% normal goat serum for 2 h at room temperature. The sections were washed thoroughly three times in PBS, then incubated with the avidin–biotin complex (Vector Laboratories, Cambridge) for a further 30 min and rinsed in PBS. Chromagens (3-amino-9-ethylcarbazole (AEC), red reaction product; or 3,3′-diaminobenzidine (DAB), brown reaction product; Vector Laboratories) were added as the final substrate, and were incubated at room temperature in the dark for 10–15 min. Each slide was then rinsed carefully with distilled water. Slides were mounted and examined by light microscopy.

The specificity of the primary antisera was tested as follows: (a) sections were incubated with antibody that had been preabsorbed with its synthetic antigen (10 μg of antigen per ml diluted antiserum) in our laboratory (Corcoran, 1996); (b) the primary antibody was omitted from the incubation; or (c) normal rabbit serum was substituted for the primary antibody. Positive controls comprised equine jejunum and feline uterus incubated with each of the primary antibodies.

The sections were examined histologically under the × 40 objective of a microscope, and localization and distribution of the immunoreactive nerves were classified depending on the density of staining assessed by subjective observation, and graded semiquantitatively as: –, not found; ±, very few; +, a very small number of fibres; ++, a small number of fibres; +++, a moderate number of fibres; ++++, a large number of fibres; and +++++, a very large number of fibres.

Results

All results are based on tissue material from 18 cyclic mares (seven oestrous mares and 11 dioestrous mares) (Table 1). At least two sections were examined from each histological block (n – 8 sections per site for each mare). A large number of adrenergic nerves immunoreactive for the catecholamine-synthesizing enzymes TH and DβH were present in all regions of the uterus. Adrenergic nerves were present at a greater density than were peptidergic nerves. There was no difference in the density or distribution of innervation between oestrous and dioestrous mares (data not shown) with subjective observation and so sections from all mares were included in one group, irrespective of stage of the cycle.

Distribution of adrenergic nerves (TH and DβH)

Nerves immunoreactive (IR) for TH and DβH were found throughout all regions of the uterus. Numerous TH-IR nerve bundles and fibres (Fig. 1a) were found in the uterine horn, body and the cervix. In the muscle layers, nerve fibres containing TH-IR often travelled parallel to the muscle fibres. Nerve fibres immunoreactive for TH were distributed along the vessels (Fig. 1b) extending from the longitudinal to the circular smooth muscle layers of the myometrium. The density of immunoreactivity for TH was greater in the myometrium than in the endometrium. Some nerve fibres entered the endometrium close to endometrial glands (Fig. 1c). Nerves immunoreactive for DβH were also present in all regions of the uterus. An intense network of nerve bundles and fibres containing DβH-IR was found in the uterine horn (Fig. 2a), body and the cervix (Fig. 2b). Nerves containing DβH were present in both myometrium (Fig. 2c) and endometrium, and were often located close to blood vessels (Fig. 2b). The density and distribution for DβH was similar to that for TH.

Distribution of peptidergic nerves (NPY, VIP, CGRP and SP)

Moderate numbers of nerve fibres containing NPY-IR were found in all regions of the uterus. Nerve fibres

Table 1. Distribution and relative density of adrenergic- and peptidergic-immunoreactive nerves in the equine uterine horn, body and cervix

Regions	Catecholamine synthesizing enzymes		Neuropeptides			
	TH	DβH	NPY	VIP	CGRP	SP
Uterine horn						
Endometrium	+++	+++	++	+	+	–
Myometrium	+++++	+++++	+++	+	+	–
Vascular layer	+++++	+++++	++++	±	±	–
Uterine body						
Endometrium	+++	+++	++	+	+	–
Myometrium	+++++	+++++	+++	+	+	–
Vascular layer	+++++	+++++	++++	±	±	–
Cervix						
Endocervix	+++	+++	++	+	+	–
Myocervix	+++++	+++++	++++	++	++	±
Vascular layer	+++++	+++++	++++	±	±	–

Eight sections were examined at each site for each mare.
CGRP: calcitonin gene-related peptide; DβH: dopamine β-hydroxylase; NPY: neuropeptide Y; SP: substance P; TH: tyrosine hydroxylase; VIP: vasoactive intestinal polypeptide.
Semiquantitative evaluation of the density of the adrenergic- and peptidergic-immunoreactive nerve fibres graded as: –, not found; ±, very few; +, a very small number of fibres; ++, a small number of fibres; +++, a moderate number of fibres; ++++, a large number of fibres; and +++++, a very large number of fibres.

containing NPY were present in both the circular and longitudinal muscle layers travelling in parallel to the long axis of the smooth muscle layers (Fig. 3a) and often associated with blood vessels forming vessel-surrounding plexiform networks (Fig. 3b). The density of NPY was greater in the myometrium than in the endometrium (Table 1). Nerve fibres containing NPY-IR were seen in the endometrium beneath the luminal epithelium, near endometrial glands (Fig. 3c) and submucosa. Nerve cell bodies immunoreactive for NPY were also observed.

The distribution of nerve fibres containing VIP-IR was similar to that of nerve fibres containing NPY-IR but there were fewer of them. A small number of scattered nerve fibres containing VIP-IR were seen in the interstitial connective tissue and along the smooth muscle layers (Fig. 4a) occasionally associated with blood vessels. In the endometrium, nerve fibres containing VIP-IR were seen around the endometrial glands (Fig. 4b). The numbers of nerves containing VIP-IR were similar in the uterine horn and body, but were higher in the cervix (Table 1).

Nerve fibres containing CGRP-IR were less numerous than nerves containing NPY, but were present in similar density to nerve fibres containing VIP. A small number of scattered nerve fibres containing CGRP were seen in the interstitial connective tissue and along smooth muscle layers (Fig. 5a) occasionally associated with blood vessels. Thick bundles of nerve fibres containing CGRP-IR were found in the cervix, travelling parallel to smooth muscle bundles (Fig. 5b). There were more nerve fibres containing CGRP-IR in the cervix than in other parts of uterus.

A few nerve fibres containing SP-IR were found only in the cervix. Nerve fibres containing SP-IR travelled parallel to the long axis of the smooth muscle bundles, close to blood vessels (Fig. 6).

Negative control sections (omission or preabsorption of primary antibody) failed to demonstrate any positive staining (Fig. 7) and positive controls showed strong immunoreactivity.

Discussion

This report is the first description of the distribution of adrenergic and peptidergic nerves in different regions of equine uterus. The present results provide immunohistochemical evidence for an abundant adrenergic innervation, as well as the presence of peptidergic innervation containing the neuropeptides, NPY, VIP, CGRP and SP, in the uterus of mares.

Previous studies have reported that the mammalian female reproductive tract receives several types of nerve fibres (Renegar and Rexroad, 1990; Melo and Machado, 1993), including the classical autonomic nervous system (sympathetic and parasympathetic), and peptidergic nerves containing various neuropeptides. Although the relative densities of different classes of nerve fibre vary among different reproductive organs and among species, their distribution patterns tend to be similar. In most species, the reproductive tract has rich adrenergic innervation associated with blood vessels. These nerves are composed of long postganglionic nerve fibres, the cell bodies of which are in the lumbar and mesenteric ganglia and short postganglionic nerve fibres originating in the pelvic ganglia (Sjöberg, 1967). These 'short' adrenergic nerves are believed to innervate predominantly the smooth muscle of the reproductive

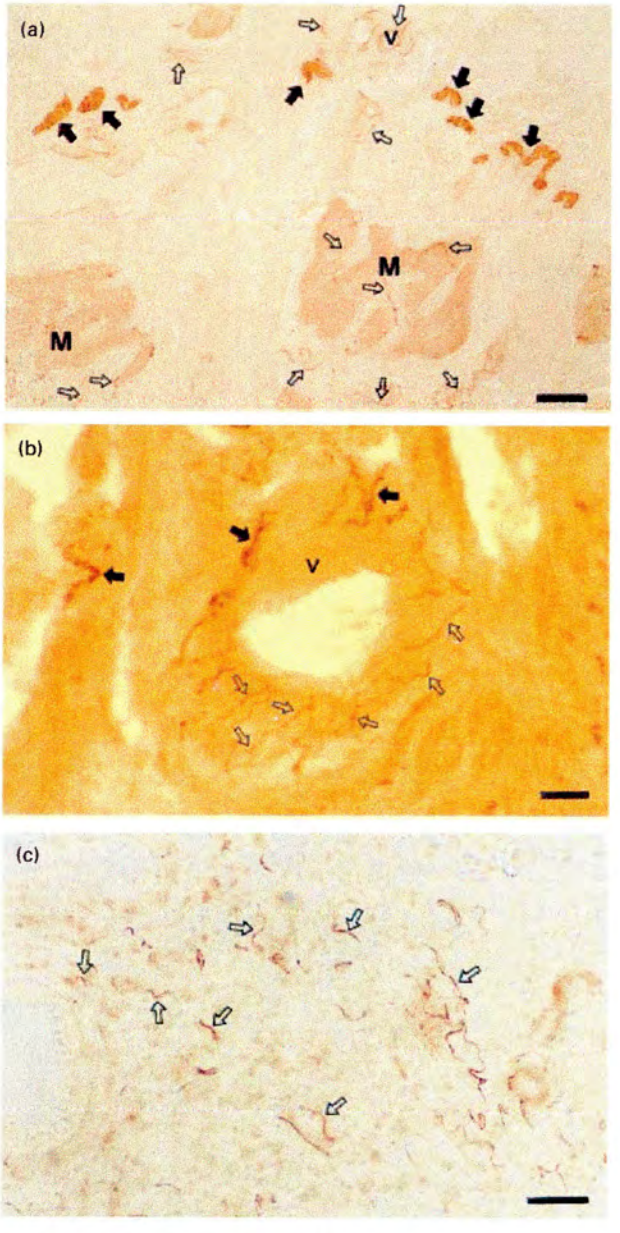

Fig. 1. Tyrosine hydroxylase (TH)-immunoreactive (IR) nerve fibres in different uterine regions in the mare. (a) TH-IR nerve bundles (solid arrows) and nerve fibres (open arrows) in the myometrium of the uterine body. M: smooth muscle bundles; V: blood vessel. (b) TH-IR nerve bundles (solid arrows) and nerve fibres (open arrows) in the myometrium of the uterine horn associated with blood vessels (V). (c) TH-IR nerve fibres (open arrows) close to the endometrial glands in the endometrium of the uterine body. Scale bars represent 250 μm (a) and 50 μm (b,c).

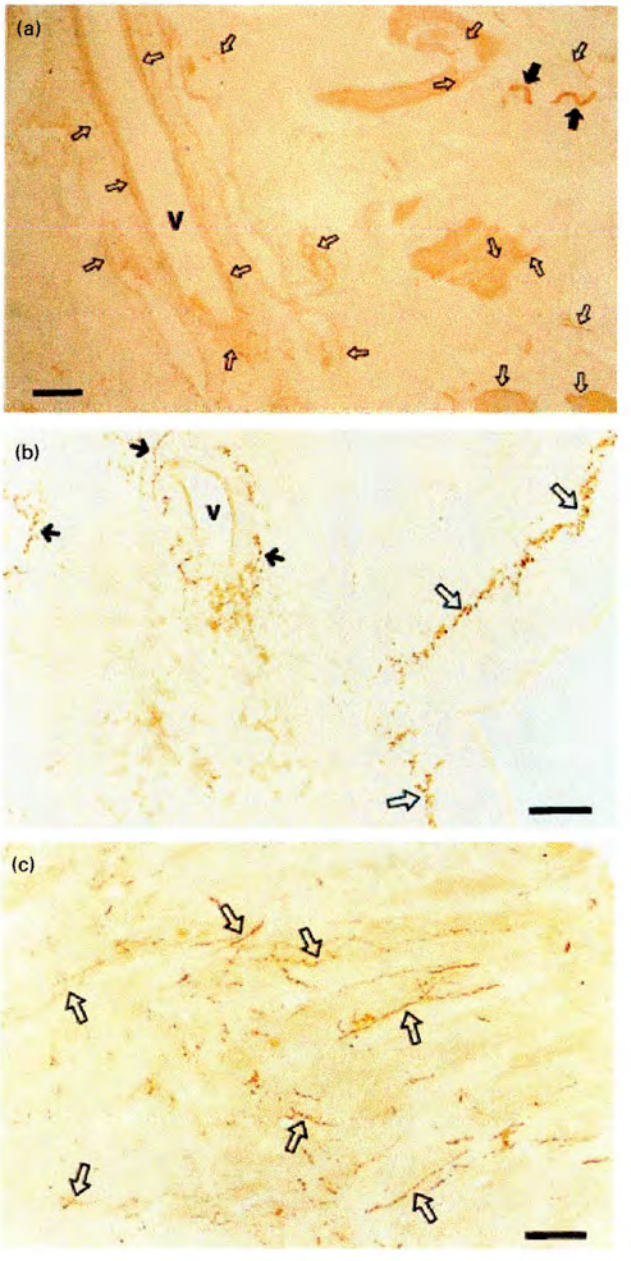

Fig. 2. Dopamine β-hydroxylase (DβH)-immunoreactive (IR) nerve fibres in different uterine regions in the mare. (a) DβH-IR nerve bundles (solid arrows) and nerve fibres (open arrows) in the myometrium of the uterine horn. V: blood vessel. (b) DβH-IR nerve fibres (open arrows) in the cervix and nerve fibres (solid arrows) associated with blood vessels (V). (c) DβH-IR nerve fibres (nerve bundles; open arrows) in the smooth muscle layers of the uterine body. Scale bars represent 250 μm (a), 50 μm (b) and 100 μm (c).

organs and they differ from the long nerve fibres anatomically. The most interesting feature of these short adrenergic nerves is the regulation of transmitter (noradrenaline) metabolism in these nerve fibres by ovarian steroid hormones (Owman and Sjöberg, 1973; Marshall, 1981). In cyclic animals, the changes in uterine innervation vary among species. Melo and Machado (1993) and Wrobel and Kujat (1993) reported that the density and distribution of the nerve fibres was nearly the same during oestrus as in dioestrus in cows, whereas in rats, Zoubina *et al.* (1998) reported that a

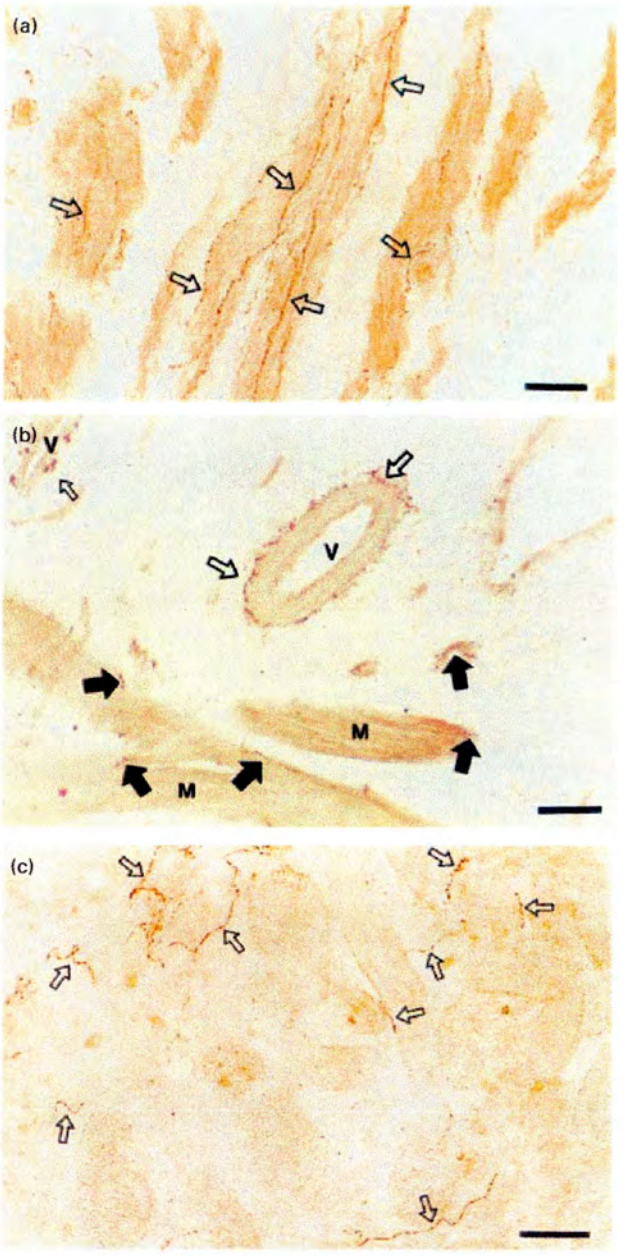

Fig. 3. Neuropeptide Y (NPY)-immunoreactive (IR) nerve fibres in different uterine regions in the mare. (a) NPY-IR nerve fibres (open arrows) in the outer longitudinal smooth muscle layer of the uterine horn. (b) NPY-IR nerve fibres (solid arrows) in the smooth muscle bundles (M) and nerve fibres (open arrows) associated with blood vessels (V) in the cervix. (c) NPY-IR nerve fibres (open arrows) close to the endometrial glands of the uterine body. Scale bars represent 50 μm.

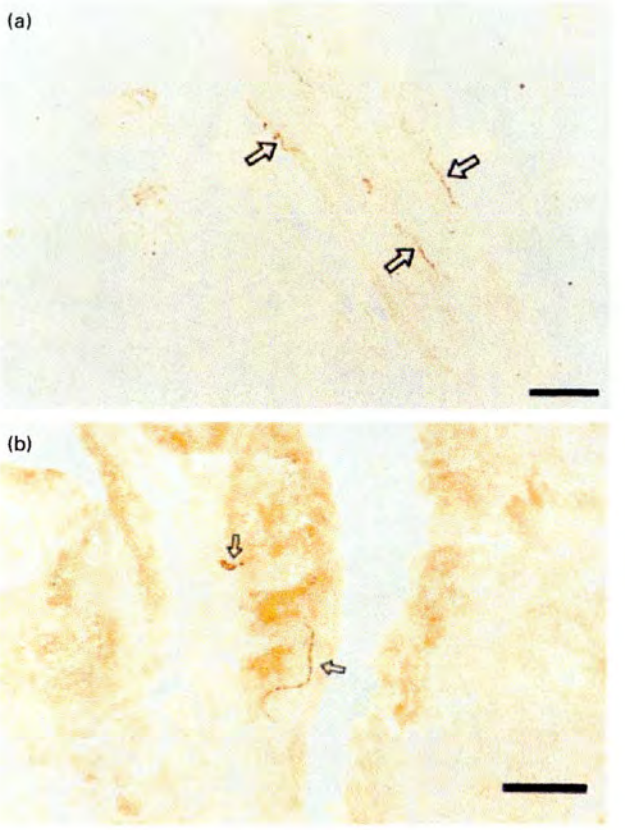

Fig. 4. Vasoactive intestinal polypeptide (VIP)-immunoreactive (IR) nerve fibres in different uterine regions in the mare. (a) VIP-IR nerve fibres (open arrows) in the myometrium of the uterine body. (b) VIP-IR nerve fibres (open arrows) in the endometrium of the uterine horn. Scale bars represent 50 μm.

reduction in the numbers of nerves occurred during oestrus and was followed by subsequent restoration of innervation. In the present study in mares, stage of cycle did not appear to influence uterine or cervical innervation.

In the present study, TH- and DβH-IR nerves had a similar distribution and both enzymatic markers were probably demonstrating the presence of noradrenaline. As in other species, intense adrenergic innervation was present in the smooth muscle layers often associated with blood vessels, and in the endometrium. In the myometrium, abundant thick nerve bundles were located with smooth muscle bundles and, in the endometrium, adrenergic nerve fibres were found in endometrial glands and near endometrial vessels, but nerve bundles were seen only rarely. These adrenergic nerve fibres associated with blood vessels, endometrial glands and myometrial smooth muscle probably function in the regulation of uterine blood flow, endometrial secretion and uterine contractility (Marshall, 1981; Renegar and Rexroad, 1990).

The distribution of the peptidergic nerves in the equine uterus was similar to that reported in guinea-pigs (Alm and Lundberg, 1988; Mitchell and Ahmed, 1992), rats (Serghini *et al.*, 1998), sows (Taneike *et al.*, 1991; Majewski *et al.*, 1995) and women (Blank *et al.*, 1986; Jørgensen, 1994). Of the neuropeptides, NPY-IR nerve fibres were particularly numerous in the equine uterus. NPY-IR nerves appeared to have fewer nerve fibre bundles than did the adrenergic

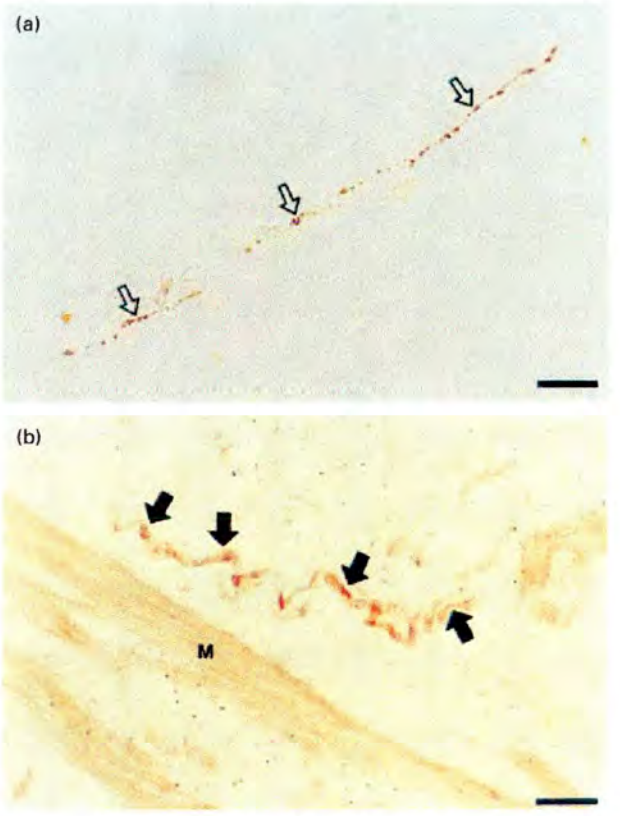

Fig. 5. Calcitonin gene-related peptide (CGRP)-immunoreactive (IR) nerve fibres in different uterine regions in the mare. (a) CGRP-IR nerve fibres (open arrows) in the myometrium of the uterine body. (b) Thin CGRP-IR nerve bundles and fibres (solid arrows) in the cervix. M: smooth muscle bundles. Scale bars represent 50 μm.

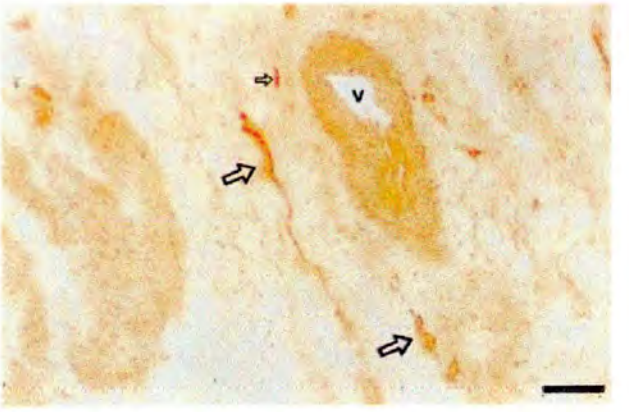

Fig. 6. Substance P (SP)-immunoreactive nerve fibres (open arrows) near blood vessel (V) in inner smooth muscle layer in the equine cervix. Scale bar represents 50 μm.

nerves, but their pattern of distribution was similar to that of adrenergic innervation. These findings indicate that most NPY-IR nerve fibres in the equine genital tract co-exist with TH-/DβH-IR nerves as documented in other species (Alm and Lundberg, 1988; Mitchell and Ahmed, 1992), but this needs to be confirmed by double-immunostaining. Previous quantitative studies have shown that NPY concentrations are greater in the cervix than in the uterine body of rats (Serghini *et al.*, 1998) and women (Blank *et al.*, 1986; Jørgensen, 1994). However, in guinea-pigs, concentrations were greater in the uterine body (Huang *et al.*, 1984). In contrast, an immunohistochemical study showed that abundant NPY-IR nerves were present in all regions in the guinea-pig genital tract (Alm and Lundberg, 1988). In the present study in mares, the density and distribution of NPY-IR nerve fibres were similar in the uterine horn, body and the cervix.

A small number of fine nerve fibres containing VIP-IR and CGRP-IR were found in the equine uterus and the density of these nerve fibres was greater in the cervical region. In the myometrium, nerve fibres containing VIP and CGRP were found in the smooth muscle layers often associated with blood vessels, and nerve fibre bundles were found only rarely. In the endometrium, nerve fibres containing VIP and CGRP were found near endometrial glands. These findings are in agreement with reports in other species (Heinrich *et al.*, 1986; Ottensen and Fahrenkrug, 1995), although there are differences in the density of distribution of the neuropeptides reported among species. In cats (Alm *et al.*, 1986), mice (Huang *et al.*, 1984) and women (Blank *et al.*, 1986), nerve fibres containing VIP-IR are abundant in the uterus, whereas guinea-pigs have only a small number (Heinrich *et al.*, 1986; Alm and Lundberg, 1988). In contrast, the density of CGRP is similar in all species. The small number of nerve fibres containing CGRP found in the female reproductive tract has been reported to be slightly denser in the cervix than in the uterine horn and body in sows (Lakomy *et al.*, 1994).

Substance P is the least frequently reported neuropeptide transmitter in the female reproductive tract (Huang *et al.*, 1984; Majewski *et al.*, 1995) and was found only rarely in the uteri of women or guinea-pigs (Heinrich *et al.*, 1986; Mitchell and Ahmed, 1992). In the present study in mares, SP-immunoreactive fibres were present in the cervix only.

Many roles have been suggested for these neuropeptides in the female reproductive tract. The density of the peptidergic nerves is less than that of adrenergic nerves, but it is clear that neuropeptides have a role in controlling female reproductive function. NPY is known to coexist with noradrenaline in most sympathetic vasoconstrictor nerve fibres (Lundberg *et al.*, 1990; Morris, 1990) and seems to have important biological functions in the regulation of vascular tone and myometrial contractility (Stjernquist and Owman, 1987; Atke *et al.*, 1996). The presence of nerves containing VIP in the female reproductive tract, as well as the dose-dependent inhibitory effect of VIP on its smooth muscle activity, indicates that VIP plays a role in the local control of smooth muscle (Helm *et al.*, 1981; Ottensen *et al.*, 1983). VIP is a potent dilator of the uterine artery (Morris and Murphy, 1988). SP is known to produce an increase in

Fig. 7. Negative control of equine uterine horn. V: blood vessel. Scale bar represents 50 μm.

myometrial blood flow and to stimulate both mechanical and myoelectrical activity (Ottensen *et al.*, 1983). CGRP and SP are present and coexist in some uterine afferent nerves and influence uterine contractility: SP has a contractile effect and CGRP has a relaxing effect (Shew *et al.*, 1991). The present results showed peptidergic nerve fibres coursing beneath the luminal epithelium and endometrial glands in the endometrium, in agreement with previous observations (Mitchell and Ahmed, 1992) and indicating a role for neurogenic mechanisms in the regulation of mucous secretion and endometrial secretion in the equine reproductive tract, as in other species (Blank *et al.*, 1986; Jørgensen, 1994; Atke *et al.*, 1996).

The presence and location of adrenergic and peptidergic innervation in the equine uterus and cervix indicate an important role for these neuronal factors in the regulation of uterine motility, endometrial secretion and blood flow. The fact that the adrenergic and peptidergic nerves are located predominantly in the myometrium indicates that neurogenic factors have an important role in controlling uterine contractility. The high density of innervation in the cervix indicates a role in regulating cervical closure in dioestrus and during pregnancy, and in relaxation of the cervix at oestrus and during parturition in mares. However, the exact functions of specific nerve fibres in the female reproductive tract remain unclear. Further studies are required to clarify their precise physiological role in the regulatory mechanisms of the equine reproductive tract.

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ELSEVIER

UTERINE CONTRACTILITY IS NECESSARY FOR THE CLEARANCE OF INTRAUTERINE FLUID BUT NOT BACTERIA AFTER BACTERIAL INFUSION IN THE MARE

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ABSTRACT

Bacteria were infused into the uteri of 5 estrous mares resistant to persistent mating-induced endometritis, first during a control cycle, and then during treatment with clenbuterol, a β_2 agonist. Uterine cellular response was evaluated 48 h later by transrectal ultrasonography, followed by uterine lavage. During clenbuterol treatment all mares accumulated intrauterine fluid, whereas in the control cycle none of the mares retained fluid. There was no significant difference between the 2 cycles in the cloudiness of the lavage fluid, number of cells per milliliter, percentage of neutrophils and frequency of bacterial growth from the recovered fluid. We conclude that uterine contractility is important in the clearance of uterine fluid, but not necessarily for the elimination of bacteria, thus supporting the published evidence that impaired uterine contractility contributes to the pathogenesis of persistent mating-induced endometritis.

Key words: mare, clenbuterol, endometritis, uterine contractility

INTRODUCTION

Endometritis is the commonest cause of subfertility and the third most common medical problem in the mare (36). Endometritis in the mare has recently been divided into four broad categories: sexually transmitted disease, chronic infectious endometritis, persistent mating-induced endometritis (PMIE) and chronic degenerative endometrosis (23,38). Susceptibility to PMIE is defined as the inability of the estrous mare to clear intraluminal fluid accumulations within 12 to 48 h after breeding (19,26,37). The primary mechanism responsible for persistence of fluid appears to be impaired physical clearance of the uterus. It has been shown that mares with PMIE have reduced electrical myometrial activity in response to intrauterine bacterial challenge (40), are less able to clear the uterus of radiocolloid (27) and other markers (12,39), possibly have lymphatic dysfunction (26) and have visibly lower uterine motility as determined by ultrasound scanning (31), than genitally normal mares. A recent study has shown that administration of phenylbutazone to block prostaglandin $F_{2\alpha}$ synthesis, one of the ecbolic

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hormones, impeded clearance of radiocolloid from the uterus (5). In order to further understand the pathogenesis of PMIE it is important to demonstrate that impairment of uterine contractility can convert a genitally normal mare into a mare which accumulates fluid after intrauterine challenge.

In the present study we used clenbuterol, a β_2 agonist, to reduce the activity of the myometrium. Beta$_2$ agonists tend to depress contractile activity of the uterus rather than cause relaxation (13) and act as antagonists to the ecbolic effects of both oxytocin and prostaglandin $F_{2\alpha}$ (10). The most important action of β_2 agonists is to close the gap junctions between myometrial cells, thereby decreasing the ability of action potentials to propagate between cells. Clenbuterol is widely used in veterinary practice as a broncholytic (10,45) and tocolytic (8,15) drug and has been used experimentally to suppress uterine contractility during early pregnancy in mares (14). In the present study we firstly investigated the effect of clenbuterol on uterine contractility and then went on to determine whether clearance of experimentally-induced intrauterine infection was impaired in genitally-normal estrous mares treated with clenbuterol.

MATERIALS AND METHODS

Animals

Five mares, weighing 320 to 500 kg and aged 4 to 11 yr, were classified as resistant to PMIE according to their reproductive history of high fertility, ability to clear introduced intrauterine infection within 48 h from the time of natural service and endometrial biopsy scores of 1 to 2A. None of the mares had any detectable intrauterine fluid accumulations prior to any of the experiments.

Experiment 1

When the mares (n=5) were detected in estrus by teasing positively to a stallion, with an ovarian follicle greater than 30 mm in diameter and uterine edema, uterine activity was monitored ultrasonographically and recorded continuously on a video cassette recorder from 5 min before until 10 min after the administration of clenbuterol (1µg/kg iv, Planipart[a]). Uterine activity was then monitored for 5 min every 2 h for the following 8 h. One minute segments of the videorecorded uterine activity were graded in a modified scale of 1 (minimum) to 10 (maximum). A similar method has been described in the past utilizing a different scoring system (6,7).

Experiment 2

In order to extrapolate volume of intrauterine fluid with ultrasound images of fluid, the mares (n=4) were given an intrauterine infusion of 6 different volumes (20, 40, 60, 80, 100, 250 ml) of sterile phosphate buffered saline (PBS) during estrus. Each infusion was performed at a different cycle after establishing that there was no existing uterine fluid. The uterus was scanned to verify

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[a] Boehringer Ingelheim Ltd., Bracknell, Berks, UK.

that all of the fluid went into the same pocket and the least possible pressure was applied to the uterus by the operator. On each occasion the area of the anechoic intrauterine fluid was calculated, within 2 min from infusion, by multiplying the maximum width and height from three different images of the two-dimensional ultrasonographic image and the mean calculated. For comparison, the mean diameter of the fluid was calculated from the height and width of the anechoic image. Ultrasonographic measurements were plotted against volume infused.

Experiment 3

When mares (n=5) teased positively to a stallion, had ultrasonographic evidence of uterine edema, and a 35-mm ovarian follicle, they were injected intravenously with 2,400 IU hCG[b] to induce ovulation within 36 to 48 h (9). On the next day the mares were given an intrauterine infusion of a bacterial inoculum (40 mL). The tail was wrapped in a plastic sleeve, the rectum was evacuated manually, and then the genital area was washed three times with tamed povidone iodine solution and dried. The inoculum was infused via a sterile insemination pipette guided by a sterile gloved hand. Forty-eight hours after infusion, the reproductive tracts of the mares were examined by transrectal ultrasonography for the presence of intrauterine fluid. The ovarian findings were recorded and quantity of uterine fluid was extrapolated from the graph generated in experiment 2 (Figure 1). The uterus was then flushed as described below. The procedure was repeated at a subsequent estrus. This time an indwelling cannula,[c] was placed in the jugular vein and clenbuterol (1µg/kg) was administered intravenously, 15 min before, and every 8 h after the bacterial infusion for the next 48 h until the uterus was flushed. An interval of 14 to 362 d (mean = 88.4±66.5 d) elapsed between the 2 treatments.

Uterine Flush

The uterus was flushed with 60 ml PBS via a catheter[d] after preparation of the genital area and the same amount of fluid was recovered into a sterile bottle. The volume of the recovered fluid was measured and the degree of cloudiness was graded visually on a scale from 0 (crystal clear) to 5 (purulent). Two well mixed 15-mL aliquots of the recovered uterine flush were processed further. The first aliquot was centrifuged at 200 x g for 15 min, after which the supernatant was carefully removed and the cell pellet was resuspended in 1 mL sterile PBS. Total cell numbers were counted using a haemocytometer. Cytospin preparations were made and stained with Diff Quik[e] and the percentage of neutrophils to epithelial cells was determined. The second aliquot was centrifuged at 2000 x g for 15 min and resuspended in 0.5 mL sterile PBS. The sample was then vortexed and streaked onto blood agar plates. The plates were then incubated aerobically at 37°C and checked for bacterial growth 24 and 48 h later.

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[b] Chorulon, Intervet, Cambridge, UK.

[c] Presidio Medico, Ecouen, France.

[d] Franklin Medical, High Wycombe, UK.

[e] Baxter Healthcare Ltd, Thetford, UK.

Preparation of the Inoculum

Streptococcus zooepidemicus was isolated from the uterus of a mare with acute endometritis and the bacteria were stored at -70°C in brain-heart infusion broth (BHIB), containing 10% glycerol. On the day before infusion, an aliquot was thawed, inoculated into BHIB and incubated overnight at 37°C. The bacteria were then washed three times in sterile phosphate buffered saline (PBS, pH 7.0) and resuspended at 750 x 10^6 colony forming units/ml by calibration of optical density. A sample of the inoculum was cultured at the time of each infusion to check the purity of the culture. Immediately prior to infusion, 1 mL of the washed bacteria was placed in 40 mL of sterile PBS.

Statistical Analysis

Uterine motility scores before and after clenbuterol administration in Experiment 1 were compared using a paired t-test. The degree of correlation between area and diameter of intrauterine fluid and volume of infused fluid in Experiment 2 were analyzed using a Spearman's rank test and its associated 95% confidence interval. Measurements made from the uterine flushes of the control and the clenbuterol-treatment group were compared using a paired t-test.

RESULTS

Experiment 1

Uterine motility prior to the administration of clenbuterol ranged from Grades 6 to 8 (Figure 1). Within the first 15-min period after the intravenous administration of clenbuterol, uterine motility decreased significantly ($P<0.05$) and resumed former levels between 6 and 8 h later.

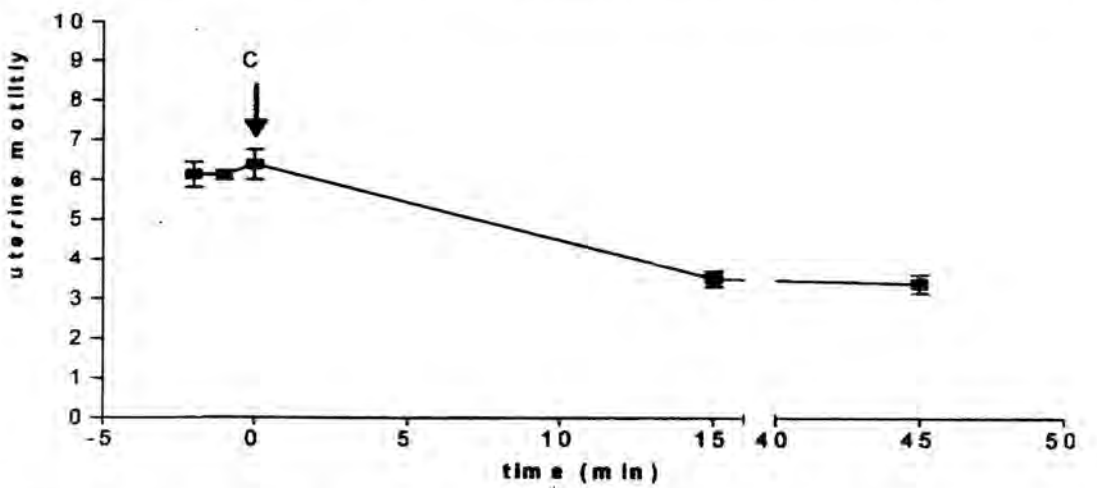

Figure 1. Uterine motility score, as detected by ultrasonography, before and after the administration of clenbuterol (C). Each point represents the mean (±SEM) of 4 values.

Experiment 2

Mean (±SEM) anechoic areas of fluid in the uterus associated with each volume of infused fluid are shown in Figure 2. Immediately after the infusion, it was seen that fluid volumes up to 100 mL had the tendency to form a single pocket of fluid located around the horn-body junction. However, measurements were not obtained from the mares after infusion with 250 mL since the fluid dispersed into several locations in the uterus. Area of the fluid was well correlated with volume infused (r=0.975), whereas the mean diameter of the non-echogenic fluid image was only poorly correlated with infused volume (r=0.465).

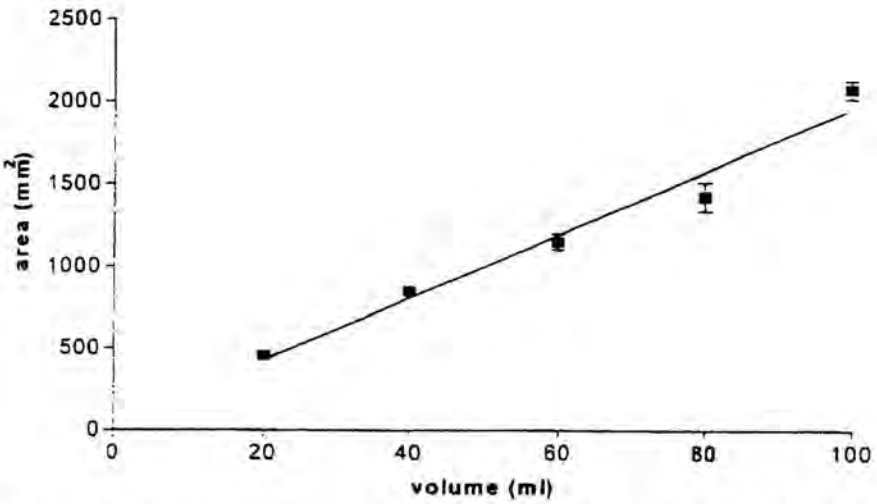

Figure 2. Ultrasonographic measurements of area (mm^2) calculated after infusion of different volumes of fluid into the uterus of the mare (r=0.975). Each point represents the mean (±SEM) of 6 values.

Experiment 3

Pure growth of Streptococcus zooepidemicus was isolated from all samples of the cultured inoculum. All mares had ovulated by the time of the uterine flush, 48 h after infusion of the bacterial inoculum. None of the mares had any uterine fluid accumulation during the control cycle but all mares accumulated fluid during the treatment cycle. Volumes were extrapolated from ultrasonographic area measurements and are shown in Table 1.

The volume of recovered uterine fluid was approximately the same (55±3.7) as the volume infused, even if there was already fluid in the uterus. Although cloudiness of recovered fluid

Table 1. Gross, cellular and bacteriological findings 48 hours after bacterial inoculation for the clenbuterol-treated (C-Tr) and the nontreated (N-Tr) group of mares

Mare	Volume of uterine fluid mL		Quality of recovered fluid[a]		Cell number recovered ($x10^4$/mL)		Bacteria isolated[b]		Percentage of neutrophils	
	N-Tr	C-Tr	N-Tr	C-Tr	N-Tr	C-Tr	N-Tr	C-Tr	N-Tr	C-Tr
1	-	77	1	1	11.3	6	-	+++	7	61
2	-	84	2	2	32	8	+	-	75	70
3	-	64	2	3	79	430	+*	+++	78	97
4	-	46	2	4	109	185	++	-	99	97
5	-	54	1	3	7	110	-	-	39	75
Mean±		65±	1.6±	2.6±	56.7±	147.8±			59.6±	80±
SEM		7.0	0.2	0.5	20.5	78.3			16.3	7.3

[a] Quality of recovered fluid was graded on a scale of 0 (crystal clear) to 5 (pus).

[b] Bacteria isolated in culture (- negative, + under 10, ++ 10-100, and +++ more than 100 colonies). Only S. zooepidemicus, was isolated except in one mare (*) where E.coli was isolated.

tended to be greater after clenbuterol treatment, this failed to reach significance)P=0.09). There was no significant difference between the control and treatment cycles in number of cells and percentage of neutrophils recovered in the uterine flush (Table 1). A heavy growth of S. zooepidemicus was isolated from 2 of the mares in the clenbuterol-treatment cycle. Two mares from the control treatment cycle had fewer than 10 colony forming units, which was probably due to contamination (17), and one had a moderate growth of S. zooepidemicus (Table 1). Within 36 h after the last clenbuterol treatment (Day 3 postovulation) none of the treated mares had detectable intrauterine fluid.

DISCUSSION

Mares which retain uterine fluid for more than 12 h after mating are considered to have PMIE (37). We have shown for the first time that inhibiting myometrial activity in genitally-normal mares results in the presence of intrauterine fluid 48 h after bacterial challenge. Thus resistant mares were effectively converted into susceptible mares.

In our mares ultrasonographic monitoring showed that uterine smooth muscle activity decreased significantly within 15 min of clenbuterol administration and the effect lasted for 6 to 8 h. This finding is similar to reports in the cow where the uterine motility decreased within 10 to 20 min (8), and parturition was postponed for 5 to 8 h (15). Although, as predicted, uterine activity was not abolished by administration of clenbuterol, it was substantially reduced. Clenbuterol is currently used at a dosage similar or higher (0.8-3.2 µg/kg b.i.d.) than that used in the present study, for long periods of time in the treatment of chronic obstructive pulmonary disease (COPD) (21). It is possible that by interfering with uterine smooth muscle activity such

therapy may predispose brood mares to the accumulation of intrauterine fluid and cause fertility problems.

The presence of a small volume of anechoic intrauterine fluid during estrus, in the absence of microbiological and cytological signs of acute inflammation, is generally not of inflammatory origin (2,33,35). However, the detection of intrauterine fluid in the uterus of the mare after breeding is usually a sign of uterine inflammation and is related to reduced fertility (1). In the quantification and scoring of intrauterine fluid accumulations in the mare, image size measurements (1,33), fluid echogenicity (30) and percentage of cases with intrauterine fluid accumulations in different uterine locations (18) have been employed. In the present study, the form of the created pocket was approximately rectangular or ellipsoidal depending on its position within the uterus and therefore its area could be calculated from the multiplication of the two readings. These area measurements correlated well with volume infused and allowed us to extrapolate volume of accumulated intra-uterine fluid. When the volume of infused fluid was correlated with the mean diameter of two readings (height and width) as it is often done in practice, statistical analysis showed only poor correlation. In our study, volumes of intrauterine fluid greater than 100 mL were usually dispersed in multiple pockets over a greater area of the uterine body and horns.

In clinical cases of PMIE the causes of the presence, distribution and retention of uterine fluid are multifactorial. Poor perineal conformation, fibrosed cervix, multiparity and age, among others (5), cause structural changes to the uterus that impair uterine contractility and lymphatic drainage, compromising uterine clearance. In older, multiparous, mares, a more flaccid and relaxed uterus extending over the brim of the pelvis is more likely to evacuate intrauterine fluid accumulations at a much slower rate than in maiden mares (28). In the present study we used young reproductively sound, maiden mares and therefore specifically addressed the role of uterine motility in expelling intrauterine fluid without the presence of confounding conformational factors.

Low-volume uterine flush as employed in this study is a technique that allows the sampling of a larger surface of the endometrium than the endometrial swab (3). The degree of cloudiness of the recovered intrauterine fluid has been positively correlated with the amount of white blood cell infiltration and is an indication of the degree of inflammation (30). In our study, cell numbers tended to be higher in the clenbuterol-treated group but this difference failed to reach significance. It is probable that number of cells per mL of recovered intrauterine fluid in the clenbuterol-treated group was diluted by the presence of existing intrauterine fluid prior to flushing. Failure to compensate for existing uterine fluid has been described as a problem in calculating the numbers of cells in uterine flushes (16).

Resistant mares usually have very few intrauterine neutrophils when the uterus is free from inflammation (4). The induction of uterine inflammation by bacterial inoculation causes neutrophil infiltration that peaks at about 6 h after the introduction of bacteria into the uterus (43,44). In the present study percentages of neutrophils in uterine lavage fluid were high in both groups, which is similar to the findings of an earlier study (32) where neutrophilia was still present 48 h after artificial insemination in both resistant and susceptible mares.

Response to artificial insemination is probably the uterine challenge that best classifies a mare as a resistant or susceptible to PMIE (32). Equine spermatozoa are chemotactic for neutrophils in vitro (38), and it has been shown that artificial insemination results in an inflammatory response in both susceptible and resistant mares, despite the addition of antibiotic to the extender (32). This emphasizes the importance of sperm in the antigenic challenge in vivo (20,32). However, it is inevitable that bacterial contamination will result from breaching the mare's cervix, and the intrauterine infusion of a bacterial inoculum in the present study provided a controlled and uniform stimulus, which has been widely used in the past (25,29,40). The numbers of bacteria recovered from the lavage fluid from the nontreated group were low to moderate. Only 1 mare in the control cycle had a significant number of bacteria in recovered uterine fluid. Two mares had fewer than 10 colonies which can be regarded as insignificant (17). However, perhaps more interestingly, heavy growth of S. zooepidemicus was observed in only 2 mares from the clenbuterol-treated group. We have previously shown that not all susceptible mares are infected with bacteria after intrauterine challenge (32), and our findings in the present study suggest that PMIE is caused more by a defect in uterine contractility than by a deficit in antibacterial properties of the uterus. Our results show that high uterine motility is not necessary for elimination of uterine bacteria and that intrauterine fluid can accumulate in the absence of infection.

The rapid timetable of bacterial clearance suggests that nonspecific antibacterial mechanisms, such as the antibacterial properties of uterine fluid and neutrophil phagocytosis, are responsible for elimination of bacteria rather than specific immunological mechanisms. The results of our study suggest that the widespread use of antibiotic infusion in mares with PMIE (34) may not be justified. Rather, the use of ecbolics alone possibly combined with uterine lavage between 6 and 12 h after breeding mares with PMIE (22,24,37) should be encouraged.

Uterine cellular and bactericidal mechanisms appear to be dysfunctional during the postovulatory period because of the suppressive effects of progesterone on uterine defense mechanisms, uterine contractility and cervical closure (11,25,41,42). However, within 36 h of the low volume uterine flush and the end of the clenbuterol treatment, all mares in the present study had cleared intrauterine fluid accumulations. Therefore, even in the presence of increasing concentrations of progesterone, once uterine contractility returned to normal, intrauterine fluid accumulations were evacuated despite the negative influence of progesterone.

In conclusion, genitally-normal estrous mares successfully expelled intrauterine fluid accumulations by 48 h after bacterial challenge whereas reduction of uterine contractility in the same mares resulted in accumulations of intrauterine fluid. It appears therefore that clenbuterol converted the resistant uterus to a susceptible uterus by inhibiting myometrial activity. Because 3 of the 5 mares with intrauterine fluid accumulations eliminated bacteria, we can conclude that bacteria can be cleared either by nonspecific antibacterial defense mechanisms or that only baseline uterine motility is necessary for bacterial elimination. These results, therefore, strongly support data from other workers showing that impaired uterine contractility contributes to persistent mating-induced endometritis in mares.

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ELSEVIER

DOES ARTIFICIAL INSEMINATION WITH CHILLED, EXTENDED SEMEN REDUCE THE ANTIGENIC CHALLENGE TO THE MARE'S UTERUS COMPARED WITH NATURAL SERVICE ?

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ABSTRACT

Uterine response in infection-resistant mares (n = 5) at 48 h after AI was compared with that following natural service in these same mares, and after AI in infection-susceptible mares (n = 6). In the resistant mares, small amounts of uterine fluid were detected and bacteria were isolated infrequently at 48 h after breeding, but cytological examination of uterine flushes revealed that a significant degree of endometritis was present. There was no difference in the degree of inflammatory response by 48 h after AI or natural mating. In 4 of the 6 susceptible mares moderate to large accumulations of intrauterine fluid were detected at 48 h after AI, and massive uterine neutrophilia was present in all 6 mares. It was concluded that there was no evidence that using conventional AI techniques limited the inflammatory response of the mare's uterus.

Key words: mare, endometritis, AI, natural service

INTRODUCTION

Susceptibility to uterine infection is the most important pathological cause of subfertility in mares. The effect of introduction of bacteria into the uterus of mares is well documented. Genitally-normal, so-called "resistant" mares will eliminate the infection within 48 to 72 h whereas susceptible mares will remain persistently infected (5, 13). Thus, the use of artificial insemination (AI) in mares that are susceptible to infection is commonly recommended to reduce antigenic challenge to the uterus (7). However, there have been no reports comparing uterine response to AI vs natural service at a time when inflammation is resolving and the uterus is preparing to receive the embryo.

In the present study, we compared uterine responses of resistant mares to AI and natural service with that of susceptible mares to AI.

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MATERIALS AND METHODS

A total of 11 mares, 5 to 16 yrs of age, were used in this study. The mares were classified as either resistant or susceptible to endometritis based on their past reproductive history. Five mares (aged 5 to 12 yr and weighing 350 to 500 kg) were classified as resistant based on the absence of uterine fluid, on <2% neutrophils present upon endometrial cytology and on endometrial biopsy Categories I to IIA (2, 8.). The remaining 6 mares (aged 8 to 16 yr and weighing 350 to 700 kg) were classified as susceptible from their reproductive history of subfertility and persistent infection after coitus. These mares had biopsy Categories of IIA to IIB. The susceptible mares were free from uterine infection (based on uterine swabbings and absence of uterine fluid) prior to insemination. All 11 mares had good vulvar and perineal conformation and a relaxed cervix at estrus.

On the day that the preovulatory follicle reached a diameter of 35 mm, as determined by transrectal ultrasonography, all of the 11 mares received the hCG[a] (2,400 IU) intravenously. On the following day (Day 1), they were inseminated artificially or were mated naturally. The mares were scanned again on Day 3 (48 h after breeding) and by this time all had ovulated.

Semen from a fertile, 5-yr-old half-breed stallion free of genital pathogens was used for all inseminations and natural breedings. This stallion had achieved pregnancy rates in excess of 80% per estrous cycle in the previous year.

Semen Collection and Preparation for AI

Semen was collected on the same day that the mares received hCG. An ovariectomized estrogen-treated mare was used as a jump mare and the stallion's penis was washed in clean warm water and dried prior to collection. The semen was prepared as described previously (23). After filtration to remove gel, the semen was diluted with Kenney's skim milk extender (containing 1 mg ticarcillin per ml) to a concentration of 25×10^6 progressively motile spermatozoa per millilitre in a total volume of 40 ml. The prepared semen (containing approximately 1×10^9 progressively motile spermatozoa) was then chilled in an Equitainer[b] for use on the next day. There was no growth from aerobic microbiological culture at 37°C for 72 h from the freshly extended semen or from extended semen chilled overnight. Fresh semen that had not been extended produced a mixed growth of nonpathogenic bacteria.

Insemination

Resistant mares were artificially inseminated and bred by natural service during different estrous cycles, whereas susceptible mares were client-owned animals whose owners would not permit their mares to be mated naturally. An interval of 26 to 62 d (mean = 41 d) elapsed

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[a] Chorulon, Intervet, Cambridge, UK

[b] Hamilton-Thorne, Beverly, MA, USA

between AI and natural service. The mares were prepared for AI and natural service in the same manner (23). Firstly, the tails were encased in plastic sleeves, then each mare's rectum was evacuated of faeces, and the perineal area and vulvar lips were washed 3 times with tamed iodine solution. The area was then dried with clean paper towels. Artificial insemination was performed using sterile equipment with a plastic surgeon's glove placed over a plastic sleeve on the operator's hand. Sterile K-Y jelly was used for lubrication.

The stallion's penis was washed with clean warm water and dried with clean paper towels prior to natural service.

Sampling Procedures

On Day 3 (48 h after AI or natural service), the mares' ovaries and uteri were scanned by transrectal ultrasonography. The occurrence of ovulation and presence of uterine fluid was recorded. The volume of uterine fluid was assessed visually by comparison with previous ultrasonographic prints of known volumes infused into the uteri of diestrous mares (data not shown). The uterus of each mare was flushed with 60 ml sterile PBS using a Foley catheter. The degree of cloudiness of the recovered fluid was noted and graded on a 5 point scale from crystal clear to pus. Then two 15-ml portions of the uterine flush were used. The first portion was centrifuged at 200 *g* for 15 min and the cell pellet was resuspended in 1 ml PBS. Cell numbers were counted using a hemocytometer, and differential cell counts were performed on cytocentrifuge preparations stained with Diff-Quik.[c] The second portion was centrifuged at 1,500 *g* for 15 min and resuspended in 0.5 ml PBS. The sample was then mixed and streaked onto blood agar culture plates undiluted and at a dilution of 1:100. The plates were incubated aerobically at 37°C and checked for growth after 24 and 48 h.

Statistical Analyses

Numbers of cells and proportion of neutrophils recovered at flush after AI and natural mating were compared using a paired t-test. Differences in these measurements between resistant and susceptible mares were analysed using a two sample t-test. Tests on cell numbers were performed on log transformed data. Frequency of recovery of bacteria from mares after AI and natural service was compared using McNemar's exact test for paired data.

RESULTS

Table 1 shows a summary of characteristics of uterine fluid recovered 48 h after AI or natural service. Resistant mares rarely had appreciable quantities of uterine fluid after either AI or natural service. By contrast, all but two of the susceptible mares had moderate to large accumulations of fluid. Quality of recovered fluid did not differ between resistant mares after AI and natural service, and was never worse than only slightly cloudy. However, fluid from susceptible mares varied from cloudy to thick and purulent. Cell numbers recovered from the fluid corresponded with visual inspection of the cloudiness of the fluid. There was no significant

[c] Baxter Healthcare Ltd, Thetford, UK.

difference between AI and natural service in the resistant mares, but a much more intense inflammatory reaction occurred after AI in the susceptible mares ($P<0.001$). The proportion of neutrophils was not different between AI and natural service in resistant mares or between resistant and susceptible mares after AI and was consistently high. Microorganisms were isolated from the uteri of 7 mares (4 susceptible and 3 resistant). The susceptible mares had yeast (n=1), mixed Steptococcus zooepidemicus and Escherichia coli (n=2) and Enterobacter spp (n=1). All of the infected resistant mares had Streptococcus zooepidemicus. Microorganisms were isolated more frequently (albeit in low numbers) from resistant mares after natural service than after AI although this did not reach significance ($0.05<P<0.1$).

Table 1 Analysis of fluid recovered from mares 48 hours after AI or natural service

Mares	Volume of uterine fluid	Quality of recovered fluid	Microorganisms isolated	No. of cells recovered($\times 10^4$/ml)	Percentage of neutrophils
Artificial Insemination					
Resistant					
1	none	crystal clear	no growth	0.2	72
2	none	slightly cloudy	no growth	21.3	76
3	none	clear	no growth	0.3	88
4	none	clear	no growth	16.3	81
5	<10ml	slightly cloudy	no growth	13.3	80
Susceptible					
6	>100ml	pus	>20 colonies	153.3	85
7	10 to 100ml	cloudy	>20 colonies	346.7	92
8	none	cloudy	no growth	686.7	79
9	>100ml	very cloudy	>20 colonies	366.7	89
10	none	cloudy	>20 colonies	81.0	90
11	>100ml	pus	no growth	152.0	63
Natural service					
Resistant					
1	<10ml	slightly cloudy	no growth	26.7	74
2	<10ml	slightly cloudy	<10 colonies	5.0	73
3	none	clear	N/D	8.0	72
4	none	slightly cloudy	>20 colonies	11.3	68
5	none	slightly cloudy	<10 colonies	3.4	76

DISCUSSION

Uterine flushes collected from resistant mares prior to intrauterine challenge contain very low cell numbers and rarely contain neutrophils (15, 22). We have shown a significant uterine response to breeding which is still present after 48 h. Cytologic evaluation of cells in recovered uterine lavage fluid has been classified as diagnostic of endometritis if more than 2% of the cells

are neutrophils (2). Therefore, all of these mares had marked residual endometritis. In the resistant mares studied, by 48 h there was no noticeable difference in the uterine response between AI, using commercially accepted procedures, and natural service despite low numbers of bacteria being isolated in flushes from some of the mares after natural service.

Neutrophil infiltration of the uterus peaks by about 6 h after experimental induction of uterine inflammation by the introduction of high numbers of bacteria (25, 27). The numbers remain elevated for at least 72 h (25) and can remain elevated for more than 10 d (27). However, there is little information about uterine inflammation after breeding. The method of collection of cells influences the number recovered. Quantitative cytological and microbiological findings of low volume uterine lavage have proved to be superior to those of endometrial swabbing (2). A recent study which used intrauterine tampons to recover uterine secretions found that neutrophils had almost totally disappeared by 48 h after AI (6). It seems likely that collection of cells by a flushing procedure would provide a more representative profile of the cells present because of contact with a larger surface area of the endometrium and the avoidance of cells adhering to the cotton tampon.

In a previous study using genitally-normal mares in which fresh rather than chilled extended semen was used, there was no difference in the number of uterine neutrophils recovered 6 h after AI or natural service (10). The presence of uterine neutrophils in resistant mares in the present study 48 h after natural breeding and insemination should be noted in view of the common practice of reinseminating mares every 48 h until ovulation. Leukocytes are known to phagocytose spermatozoa (12) and to produce substances such as oxygen-free radicals which are cytotoxic (21). However, in the postpartum period, pregnancy rates in mares do not appear to be affected by detection of neutrophils in endometrial or vestibular smears (9, 16), and studies in rabbits have shown that spermatozoa can pass through the genital tract and fertilize the ovum even in the presence of high numbers of neutrophils from an earlier mating (18).

High numbers of neutrophils persisted in our study despite there being no growth of uterine pathogens from 6 of the flushings of the resistant mares after insemination or natural breeding, and fewer than 10 colonies were isolated from 2 of the remaining flushes. Hinrichs et al. (3) suggested that the growth of fewer than 10 colonies from uterine swabs represents contaminants or transient organisms. Kotilainen et al. (10) demonstrated that either no bacteria or only very low numbers were recovered 6 h after insemination. Our study confirms the conclusions of Kotilainen et al. (10) that the intensity of the neutrophil reaction is dependent on the presence of spermatozoa and further establishes that bacterial populations do not increase after the 6-h sampling. A recent in vitro study has shown that equine spermatozoa, but not seminal plasma are chemotactic for neutrophils (20), confirming the involvement of sperm antigens in post-service uterine neutrophilia. However, bacterial contamination of the uterus is an inevitable consequence of breaching the mare's cervix, even when sterile substances or semen along with extender and antibiotic are infused, which also undoubtedly contribute to initial inflammation post insemination. Indeed, even transrectal manipulation of the cervix and uterus causes transient neutrophilia (27).

Detection of intrauterine fluid by ultrasonography is associated with uterine inflammation (1). None of the resistant mares in our study had large accumulations of intrauterine fluid 48 h after AI or natural breeding. Three mares, however, had a thin line of fluid that was not associated with isolation of significant numbers of bacteria from the uterine fluid. Differences in volumes and total (in contrast to progressively motile) numbers of spermatozoa inseminated between AI (40 ml; mean = 2.5 $x10^9$ sperm) and natural service (mean ejaculate volume for this stallion = 83 ml; 7.7 x 10^9 sperm) did not appear to influence uterine response. Probably the two to three-fold difference in volume and sperm numbers between natural service and AI was not sufficient to cause a significant difference in the inflammatory response.

In our present study, the intense neutrophilia and uterine fluid accumulation in susceptible mares after AI was similar to results of previous studies upon infusion of sterile substances into the uterus (11, 24). However, ours appears to be the first study reporting detailed uterine response to AI in susceptible mares. All but 2 of the susceptible mares accumulated ultrasonically detectable fluid by 48 h post AI, and the recovered lavage fluid from all of these mares was cloudier than that in any of the resistant mares, reflecting the higher numbers of cells. Bacteria were not isolated from the lavage fluid from two of the mares despite the presence of high numbers of neutrophils. This may have been due to the presence of infective agents such as mycoplasmas or anaerobes which were not cultured using our aerobic culture procedures, or to elimination of infection by 48 h albeit with persisting sterile inflammation, presumably due to the continued presence of antigenic material.

In resistant mares, we were unable to show any advantage of AI compared with natural service in terms of uterine inflammatory response. This was unexpected in view of the numbers of bacteria introduced directly into the uterus with semen at natural service (approximately 0.5 million/ml [17]) compared with the inseminate at AI (no aerobic micro-organisms were cultured from the extended semen in the present study, as was previously reported by Kenney et al [7]) and the presence of low numbers of bacteria in the uteri of some of the mares after natural service. Only very low numbers of anaerobes have occasionally been isolated from samples of extended semen (4), and, therefore, it is unlikely that anaerobes contributed to the inflammatory response. Perhaps this finding emphasizes the importance of nonbacterial antigens in post-service uterine neutrophilia.

It is not clear from our study that AI offers real advantage over natural service to susceptible mares, especially since we have shown that AI in susceptible mares resulted in severe inflammatory response and persistent endometritis. However, many different factors are operative in susceptible mares which makes a direct comparison of breedings between resistant and susceptible mares impossible. For example, uterine contractility appears to be impaired in some susceptible mares (11, 14, 19). In addition, changes in lymphocyte subpopulations are present in the endometrium of susceptible mares (26), which may imply that in some mares distinct immunological factors are present that may contribute to susceptibility to infection. However, bacteria, albeit in low numbers, were isolated more frequently from resistant mares after natural service than after AI. Therefore, it would seem that minimizing bacterial

contamination through AI would be the wise course in susceptible mares along with other pre- and post- AI therapies.

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Effect of infusion volume and sperm numbers on persistence of uterine inflammation in mares

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Keywords: horse; insemination; uterus

Introduction

It has now been well documented that spermatozoa, rather than bacteria, are responsible for the acute inflammatory response in the equine uterus after insemination (Kotilainen *et al.* 1994; Troedsson *et al.* 1995b; Nikolakopoulos and Watson 1997). Spermatozoa themselves are chemotactic for equine neutrophils (Troedsson *et al.* 1995b) and frozen semen inseminations which use a low volume of highly concentrated sperm produce a greater uterine response than insemination with higher volumes containing lower concentrations (Kotilainen *et al.* 1994). Seminal plasma *per se* tends to suppress neutrophil migration (Troedsson *et al.* 1997) and, therefore, inseminates containing a greater proportion of seminal plasma, such as extended chilled semen, should induce a less intense inflammatory response. There is no information, however, on degree of uterine inflammation using larger volumes of inseminate containing different concentrations of sperm.

In the present study, the uteri of mares were infused with a standard volume (40 ml) of buffer, of semen extender containing 2 or 20 billion sperm, or a large volume of buffer (500 ml). The inflammatory response was compared with that of control mares in order to investigate the effect of these different treatments on persistence of endometritis after breeding.

Materials and methods

Animals

A total of 13 mares, age 4–12 years, were used. They were classified as resistant to persistent mating-induced endometritis based on their past successful reproductive history, absence of uterine fluid, endometrial biopsy scores of I to IIA (Kenney and Doig 1986) and ≤ 5% neutrophils present in stained endometrial cytology preparations. All mares had good vulvar and perineal conformation and a relaxed cervix at oestrus.

When the mare responded positively to teasing, and uterine oedema and a large ovarian follicle were present, the mare was considered to be in oestrus. Ovulation was detected ultrasonographically by the disappearance of the follicle and the presence of a *corpus haemorrhagicum* on the ovary.

On the first day of oestrus that the mares had an ovarian follicle of at least 35 mm, they received human chorionic gonadotrophin (2400 iu hCG[1]) i.v. On the following day (Day 1) the uterus was infused with one of 4 different treatments. A variable number of the 13 mares (4–7 per group) received 2–4 of the infusion treatments in random order. These mares received only 1 treatment during each oestrus and each mare received each treatment only once. There was a minimum of 14 days between treatments. Mares were infused with: 1) 40 ml sterile phosphate buffered saline (PBS; pH 7.0; n = 4); 2) 500 ml sterile PBS (n = 5); 3) 20 x 10^9 frozen-thawed spermatozoa (n = 4); or 4) approximately 2 x 10^9 spermatozoa in 40 ml skim milk semen extender (n = 7). Six mares were not infused and served as a control group.

Semen collection and preparation of spermatozoa for infusion

Semen was collected on the same day that the mares received hCG. An ovariectomised oestrogen-treated mare was used as a jump mare and the stallion's penis was washed in clean warm water and dried prior to collection. The semen was prepared as described previously (Nikolakopoulos and Watson 1997). After filtration to remove gel, the semen was diluted with Kenney's skim milk extender containing 1 mg ticarcillin/ml (Kenney *et al.* 1983) to a concentration of 50 x 10^6 progressively motile spermatozoa/ml in a total volume of 40 ml. Approximately 50% of the spermatozoa were progressively motile. The prepared semen (treatment 4; containing approximately 2 x 10^9 spermatozoa) was then chilled in a Equitainer[2] for use on the next day. There was no growth from aerobic microbiological culture at 37°C for 72 h from the freshly extended semen chilled overnight.

For the infusion of frozen-thawed spermatozoa (treatment 3), the semen was filtered and the seminal plasma was separated from the spermatozoal fraction by centrifugation at 2,500 *g* for 20 min. The pelleted sperm was then suspended in 4 ml sterile PBS and stored at -20°C. A dose of 20 billion sperm was used, made up to a spermatozoa concentration of 500 x 10^6 spermatozoa/ml in 40 ml with sterile PBS containing ticarcillin (1 mg/ml) immediately prior to infusion. Because of the large numbers of sperm required for this infusion, it was necessary to pool several ejaculates from the stallion. Spermatozoa from each ejaculate were therefore frozen to ensure preservation of surface antigens.

Treatments

The mares were prepared for insemination and the other

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treatments using hygienic procedures as described previously (Watson 1995). All infusions were performed using sterile equipment with a surgeon's glove placed over a plastic sleeve on the operator's hand. Sterile K-Y jelly[3] was used for lubrication. Treatments were in random order for each mare and one to 2 cycles intervened between each treatment. All mares were used in more than one treatment except for one mare which was used only for AI. To ensure that any chronic irritation caused by the treatments would be detected, the control cycle was the last occasion on which a uterine flush was collected from all mares.

Sampling procedures

On Day 3 (48 h after infusion), the mares' ovaries and uteri were scanned by transrectal ultrasonography. The occurrence of ovulation and presence of uterine fluid was recorded. The uterus of each mare was flushed with 60 ml sterile PBS using a plastic Foley catheter. The volume of fluid recovered was approximately the same as that infused, and never exceeded 60 ml. Two 15 ml portions of the recovered uterine flush were used. The first portion was centrifuged at 200 *g* for 15 min and the cell pellet resuspended in 1 ml PBS. Cell numbers were counted using a haemocytometer and calculated as cells/ml of the 60 ml flush, and differential cell counts were performed on cytocentrifuge preparations stained with Diff-Quik[4]. The second portion was centrifuged at 2,500 *g* for 15 min and resuspended in 0.5 ml PBS. The sample was then mixed and streaked onto blood agar culture plates. The plates were incubated aerobically at 37°C and checked for growth after 24 and 48 h.

Statistical analysis

A one-way ANOVA, with least significant mean comparisons where appropriate, was performed to evaluate effects of treatment on neutrophil numbers in the recovered flush. A P value of <0.05 was considered significant.

Results

All mares had ovulated by the time of the uterine flush, 48 h after the intrauterine infusion. Only on 5 of 20 occasions after treatment was a thin line of fluid seen in the uterus. The presence of a trace of fluid was unrelated to type of treatment and probably represented less than 10 ml volume. Quality of recovered fluid did not differ between treatment and control groups and was never worse than slightly cloudy. No microorganisms were isolated from the uterine flushes of any of the treatment groups. Treatment had a significant effect (P<0.01) on numbers of neutrophils recovered (Table 1). Control mares had very few neutrophils present in their uterine flush but these numbers did not differ significantly from numbers recovered after infusion of 20 billion sperm or 500 ml PBS. Infusion of 40 ml PBS and 2 billion sperm in semen extender resulted in the greatest residual inflammatory response and neutrophil numbers were significantly greater than the other treatments. Percentage of neutrophils recovered in the flush in general reflected neutrophil numbers (Table 1). The remaining cells in the cytospin preparations were epithelial cells.

TABLE 1: Neutrophil recovery (mean ± s.e.) from uterine lavage of mares 48 h after various intrauterine infusions

Treatment	No. mares	Percentage neutrophils	No. neutrophils* (x 10^4/ml)
Control (no infusion)	6	2.0 ± 0.97	0.17 ± 0.15[a]
40 ml PBS	4	66.3 ± 9.07	14.8 ± 4.36[b]
AI (40 ml extended semen containing 2 x 10^9 sperm)	7	79.4 ± 2.37	11.2 ± 3.85[b]
Spermatozoa (40 ml PBS containing 20 x 10^9 spermatozoa)	4	37.0 ± 12.75	2.5 ± 0.87[a]
500 ml PBS	5	36.6 ± 11.70	2.4 ± 1.49[a]

Different superscripts denote significant differences (P<0.05).

*Number of neutrophils was calculated from the number of cells present per ml of the 40 ml flush (counted using a haemocytometer) x percentage of neutrophils in a stained cytospin preparation of the recovered fluid.

Discussion

Uterine flushes recovered from our mares during the control cycle yielded only very low numbers of cells and a low percentage of neutrophils. Low volume flush was used in preference to swabbing techniques because a greater area of the endometrium is sampled and the results have been shown to be more reliable (Ball *et al.* 1988). Our results are in agreement with other workers who classify fewer than 2–10% neutrophils as representative of a normal endometrium (Asbury 1982; Ball *et al.* 1988). Because the control cycle was the last intervention to which these mares were subjected, it appears that the other treatments had no carry-over effects.

In the present study, we found that infusion of a standard dose and volume of extended semen or PBS caused much greater residual uterine inflammation 48 h after infusion than did a large volume of buffer or high numbers of sperm. It is surprising that a similar degree of inflammation was caused by infusion of PBS and extended semen. Infusion of saline can be irritant due to low pH (Pascoe *et al.* 1989) but, in the present study, the PBS was buffered to pH 7.0. Also the fluid was sterile at infusion and no microorganisms were cultured from the recovered flush. It is, therefore, unlikely that substantial infection was introduced with the PBS. In a previous study by Kotilainen *et al.* (1994) infusion of 30 ml PBS caused less inflammation than 40 ml extended semen containing approximately 2 billion sperm. However the difference in neutrophil numbers was not great (mean ± s.e. 1.4 ± 1.3 x 10^6/ml with PBS vs. 5.0 ± 4.4 x 10^6/ml with semen). Therefore, perhaps with relatively low concentrations of sperm, it is the volume and presence of fluid in the uterus that is irritant and the chemotactic effect of the sperm is less important.

There was a relatively minor degree of inflammation after infusion of 500 ml PBS. It seems probable that this is due to the strong contractions induced in the uterus after infusion of this large volume. Expulsion of fluid in some mares through the relaxed oestrous cervix was seen almost immediately after infusion. In a previous study, the percentage of fluid refluxed did not vary with infused volumes of 30 and 250 ml (Jones 1995). However, in that study fluid loss was significantly greater in

oestrus than dioestrus and the volume used in the present study was considerably greater than that used by Jones (1995). High insemination volumes containing relatively low concentrations of sperm have resulted in reduced pregnancy rates (Squires *et al.* 1989) and those authors suggested that this was caused by cervical reflux although the low sperm concentration may have contributed to the fall in fertility (Bedford and Hinrichs 1994). In the present study, as a result of uterine distension, the fluid could have been forcefully expelled before there was time for it to have an irritant effect on the endometrium.

The low level of residual inflammation after infusion of 20 billion sperm is less easy to explain. The sperm had been frozen without cryoprotectant and would therefore have lysed. However, freezing should not have destroyed the antigens responsible for their chemotactic properties. That the sperm were dead has been shown to have no effect on degree of uterine inflammation (Katila 1997). In a previous paper, high sperm concentrations in 2–12 ml caused an intense inflammatory reaction (Kotilainen *et al.* 1994). Sperm concentration, in our study, was approximately the same as in this latter study but a greater volume was used and so approximately 20 times more sperm were infused. In our study, uterine flush was collected 48 h after infusion when the mares had already ovulated. Time of maximum uterine inflammation after insemination is around 8 h (Katila 1995; Bergman and deKruif 1997) and in our study time of major neutrophil influx had therefore passed. It could be that intense early inflammation resulted in release of prostaglandins (Watson *et al.* 1988) with concomitant uterine contraction (Troedsson *et al.* 1995a) and early resolution of inflammation. Interestingly, Troedsson *et al.* (1995b, 1997) have shown that seminal plasma can suppress sperm-induced neutrophil chemotaxis *in vitro*. The present study indicates *in vivo* that increased volume may also be a factor involved in depressing neutrophil migration into the uterus. If this proves to be true, it is possible that increasing the volume at time of inseminating frozen semen could decrease the intense inflammatory response. In our study we did not investigate the effect of skim milk semen extender compared with PBS in eliciting an inflammatory response, but there has clearly been shown to be no difference between the 2 fluids (Kotilainen *et al.* 1994).

In conclusion, in the uterus of the genitally normal mare it appeared that the more minor the insult, the greater the degree of residual inflammation 48 h later. Mares were short-cycled without being checked for pregnancy. However, even with this level of inflammation, most of these mares would have become pregnant to the insemination which comprised a standard volume of inseminate and number of sperm infused at an appropriate time prior to ovulation. As it is considered that resolution of inflammation is necessary for survival of the embryo, this indicates that under normal circumstances this degree of inflammation would have resolved within 5 or 6 days after insemination, at the time of descent of the embryo into the uterus.

Manufacturers' addresses

[1]Chorulon, Intervet, Cambridge, UK.
[2]Hamilton-Thorne, Beverly, Massachusetts, USA.
[3]Johnson and Johnson, Maidenhead, Berks, UK.
[4]Baxter Healthcare Ltd., Norfolk, Thetford, UK.

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Post-breeding endometritis in the mare

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Post-breeding endometritis in the mare

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Abstract

Post breeding endometritis is a major cause of subfertility in the mare. Endometritis is a normal event in the immediate period after mating, but the presence of ultrasonographically visible uterine fluid more than 12 h later is thought to be evidence of uterine pathology. In mares that are free of venerally transmitted endometritis, treatment is aimed at removing the intraluminal fluid. If the endometritis persists past day 5, when the embryo enters the uterine lumen, the cytotoxic environment will not be compatible with pregnancy. Reproductive anatomy, defective myometrial contractility, lowered immune defences, overproduction of mucus, inadequate lymphatic drainage, or a combination of these factors will predispose the mare to post breeding endometritis.

Keywords: Mare; Uterus; Endometritis

1. Introduction

Fertility varies markedly among mares. Apart from poor management and incorrect timing of mating, probably the most important reason for low pregnancy rates is endometritis/endometrosis. A survey of 1149 veterinarians in the United States ranked endometritis as the third most frequently occurring medical problem in adult horses (Traub-Dargatz et al., 1991). Until relatively recently, mares were classified as resistant or susceptible to endometritis based on their ability to eliminate uterine infection within a certain period of time after challenge (Hughes and Loy, 1969; Petersen et al., 1969) and/or on their endometrial biopsy scores (Kenney and Doig, 1986). Mares with high biopsy scores which had evidence of inflammatory cell infiltration or endometrial fibrosis, or mares which failed to eliminate uterine infection were referred to as

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susceptible to endometritis. However, the multifactorial nature of the condition has now resulted in subdivision of endometritis into four categories, based on aetiology and pathophysiology (Troedsson et al., 1995; Troedsson, 1997b). These are: (1) endometrosis (chronic degenerative endometritis), (2) sexually transmitted diseases, (3) persistent mating-induced endometritis (PMIE), and (4) chronic infectious endometritis. These categories are not, however absolute and mares may change categories between or even within breeding seasons, or may fit into more than one category.

2. Endometrosis

Endometrosis is a chronic degenerative condition of the endometrium and is thought to be irreversible (Kenney and Doig, 1986; Allen, 1993). It is not a post-breeding condition and will not be dealt with in this review, although severe changes can be associated with delayed uterine clearance (Troedsson et al., 1993a) and may result from repeated uterine inflammation or ageing (Allen, 1993).

3. Sexually transmitted diseases

Sexually transmitted diseases are those acute infections which are induced after mating mares with stallions which are inapparent penile carriers of *Taylorella equigenitalis*, certain unspecified serotypes of *Pseudomonas aeruginosa*, and *Klebsiella pneumoniae* capsule types 1, 2 and 5 (Platt et al., 1977). *T. equigenitalis* is a highly contagious pathogen which caused infertility in Thoroughbred mares in the UK in 1977 (Powell et al., 1978). When first diagnosed the organism caused severe acute endometritis with copious vaginal discharge in infected mares along with short oestrous cycles (Ricketts et al., 1977; Pierson et al., 1978), but recently, a more insidious form has been recognised in continental Europe which is associated with minimal clinical signs (Ricketts et al., 1992). Since its first isolation, rigorous control measures have been instituted in the UK (reviewed by Ricketts, 1996; Watson, 1997) and outbreaks of the disease are now only sporadic. *T. equigenitalis* is sensitive to several antibiotics and treatment is highly successful (Ricketts, 1996). *P. aeruginosa* and *K. pneumoniae* tend to be much more intractable to treatment (Ricketts, 1999; Wingfield Digby, 1999).

4. Persistent mating-induced endometritis

Endometritis is a normal physiological event after mating, but if the inflammation persists, the resulting environment is not compatible with establishment of pregnancy. The inflammation is often, but not always, accompanied by accumulation of intrauterine fluid. Intrauterine fluid accumulation in mares was first reported by Knudsen (1964) using rectal palpation, however more recently transrectal ultrasonography has been employed to detect intrauterine fluid (Ginther and Pierson, 1984; Adams et al., 1987; Allen and Pycock, 1988; Allen, 1991; Pycock and Newcombe, 1996b).

Accumulation of intrauterine fluid in dioestrus is associated with a significant reduction in pregnancy rates and an increase in embryonic loss rate (Adams et al., 1987). In this study, the intrauterine fluid collections were shown to represent endometrial inflammation, as the mares had high biopsy scores and shortened luteal phases. The incidence of intrauterine fluid one to two days after natural breeding in Thoroughbred mares has been reported as 15% of 746 cycles (Zent et al., 1998) and as high as 43% of 552 cycles in a mixed population of mares (Newcombe, 1997). Pregnancy rates in this latter study were 49% in mares with intrauterine fluid within 48 h after mating compared with 62% in mares without fluid.

The importance of fluid accumulation during oestrus is less clear. Between 11% and 39% of mares accumulate fluid at oestrus (Pycock and Newcombe, 1996b; Reilas et al., 1997). Squires et al. (1989) have reported that oestrous fluid reduces spermatozoal motility in vitro and reduces embryo recovery rates. By contrast in another study which also showed that spermatozoal motility was adversely affected by intrauterine fluid collected at oestrus, embryo recovery rates were not affected (Reilas et al., 1997). Although pregnancy rates were reduced by intrauterine fluid at oestrus in a further report (Pycock and Newcombe, 1996b), fluid collected from most of the mares had negative bacteriology and cytology results and therefore was not of inflammatory origin (Pycock and Newcombe, 1996b; Reilas et al., 1997). Oestrogen acts on the uterus during oestrus to increase endometrial secretion and oedema. It is thought that mares which accumulate fluid in the uterus during oestrus have more glands with larger diameter and wider lumens than mares without intrauterine fluid (Rasch et al., 1996). This might suggest that hypersecretion of mucus contributes to intrauterine fluid accumulation. However, drainage of fluid via the cervix and lymphatics is also important and mares which accumulate intrauterine fluid can have cervical fibrosis or anatomical changes often associated with old age resulting in a pendulous or ventrally tilting uterus (Le Blanc et al., 1998). Alternatively, lymphatic drainage may be impaired (Le Blanc et al., 1995).

It is now thought that a primary defect in myometrial contractility may contribute to PMIE. Delay in uterine clearance of bacteria and inert, non-antigenic material was first reported in association with progesterone treatment and increasing age by Evans et al. (1986). These authors suggested that physical clearance might play an important part in resistance of mares to uterine infection (Evans et al., 1987). The existence of delayed uterine clearance was confirmed by Troedsson and Liu (1991) using non-antigenic markers. In another study these same workers reported that susceptible mares accumulated six times more fluid in the uterus after bacterial challenge than did normal mares (Troedsson and Liu, 1992). A further study showed that reproductively normal mares had cleared $>50\%$ of a radiocolloid infused into the uterus at oestrus within 2 h, whereas susceptible mares cleared negligible amounts by 4 h (Le Blanc et al., 1994). Although the precise mechanisms underlying the inability of the oestrous uterus to clear this fluid are as yet unknown, it appears that myoelectrical activity in response to inflammation is impaired in these mares (Troedsson et al., 1993b).

Visual analysis of video recordings has also been used to study myometrial contractility in mares (Cross and Ginther, 1988) and recently it has been reported that there are significant visual differences in uterine motility between genitally normal mares and mares which show delayed uterine clearance (Nikolakopoulos and Watson, 1997b).

Although no differences were detected in plasma concentrations of ecbolic hormones in normal mares and mares with PMIE around the time of insemination, administration of oxytocin to oestrous mares susceptible to PMIE resulted in a markedly reduced release of $PGF_{2\alpha}$ compared with reproductively normal mares (Nikolakopoulos et al., 2000). Furthermore, inhibition of PG release with phenylbutazone has resulted in delayed clearance of radiocolloid in reproductively normal mares (Cadario et al., 1995). This has led to the suggestion that there may be a difference in these two groups of mares at the level of the uterine oxytocin receptor, or post-receptor mechanisms (Nikolakopoulos et al., 2000). There is recent evidence that endometrial oxytocin receptor concentrations do not differ between genitally normal mares and mares with PMIE (Cadario et al., 1998), however, myometrial receptors were not measured in this latter study.

Although the uterus depends on both hormonal and neural mechanisms, there is very little information on neurological control of myometrial contractility in the mare. In vitro studies to date have been confined to normal mares and have shown that the myometrium responds to electrical field stimulation, which induces the release of acetylcholine, but not nitric oxide. However, the myometrium was capable of responding to the inhibitory actions of exogenous nitric oxide (Liu et al., 1997). Another recent study has shown that the equine uterus is supplied mainly by adrenergic nerves. Of the peptidergic neuropeptides, neuropeptide Y was the most abundant (Bae et al., 1999). Studies now need to be extended to mares with PMIE. Further confirmation of the importance of neurologic control of myometrial contractility has recently been obtained by administration of clenbuterol, a β_2 agonist, to mares which suppressed uterine contractility and predisposed normal oestrous mares to intrauterine fluid accumulation after bacterial challenge (Nikolakopoulos and Watson, 1999).

Not only has evacuation of the uterus after mating been shown to be defective in mares with PMIE, but sperm transport to the oviduct is also affected. Mares with chronic endometritis had fewer sperm in the caudal isthmus than normal mares and very few of these sperm were motile (Scott and Liu, 1997).

Regardless of whether mares are bred by natural mating or artificial insemination, breaching of the cervix at breeding results in an equally intense inflammatory response in genitally normal mares (Nikolakopoulos and Watson, 1997a). Although many bacteria will inevitably be introduced at this time, it has recently been shown that uterine inflammation is induced at breeding in the absence of bacterial contamination (Kotilainen et al., 1994; Nikolakopoulos and Watson, 1997a). A detailed study has shown that spermatozoa themselves are chemotactic for equine neutrophils in vitro (Troedsson et al., 1995) possibly via activation of complement and these results have been confirmed in vivo (Katila, 1997). By contrast seminal plasma inhibits neutrophil migration and phagocytosis in vitro (Scheytt and Gilbert, 1995; Troedsson et al., 1995) and therefore the presence of seminal plasma may modulate inflammation allowing the spermatozoa safe passage through the uterus to the oviducts. Volume of inseminate may also influence persistence of uterine inflammation and a recent study has shown that larger volumes decrease the inflammatory response (Nikolakopoulos and Watson, 2000). In reproductively normal mares, this post-breeding endometritis is transient and subsides within 48–72 h (Hughes and Loy, 1969; Petersen et al., 1969). However, if intrauterine fluid is present at 12 h or more after mating, the mare is considered to have PMIE

(Troedsson, 1997a). Components of the intrauterine fluid include inflammatory mediators, neutrophils and plasma proteins including immunoglobulins, complement and enzymes (Watson et al., 1987a,b; Katila et al., 1990; Pycock and Allen, 1990; Troedsson et al., 1993c). These components are increased within 30 min to 12 h of uterine insult (Watson et al., 1987b; Katila et al. 1990; Pycock and Allen 1990) and speed of mobilisation of the phagocytic influx does not seem to differ between genitally normal mares and mares susceptible to endometritis (Asbury et al., 1982; Watson, 1986). However, in susceptible mares, the neutrophils remain at high numbers, whereas they decline sharply in normal mares (Katila, 1995; Nikolakopoulos and Watson, 1997a). Interestingly, there appears to be little correlation between moderate histological endometrial categories and delayed uterine clearance (Troedsson et al., 1993a). Previous work has shown that chronic inflammatory cells, one of the determinants of endometrial category, are normal components of the equine endometrium and are not necessarily indicative of endometritis (Watson and Dixon, 1993; Watson and Thomson, 1996).

5. Chronic uterine infection

Clinical experience would show that mares which are affected by PMIE initially in the breeding season, can develop into mares with chronic uterine infection. Alternatively, mares which have no previous history of PMIE can present with a uterine infection. The major pathogens involved in equine endometritis are *Streptococcus zooepidemicus*, *Escherichia coli*, or yeasts (Dimock and Edwards, 1928) although anaerobes may play a role (Ricketts and Mackintosh, 1987). In the USA, *P. aeruginosa* and *K. pneumoniae* are relatively common isolates (Dimock and Edwards, 1928) but in the UK they are relatively rare (Ricketts et al., 1992). Of these organisms, *S. zooepidemicus* is by far the most common and accounts for around 66% of infections. This organism is part of the normal microflora of horse skin and is a common contaminant of the uterus after mating. Whether infection is established or not depends on the efficacy of the mare's uterine defence system. *E. coli* is more frequently recovered from mares with anatomical defects of the perineal and vulvar region which predispose mares to pneumovagina and faecal contamination (Le Blanc 1997). Le Blanc (1997) also suggests that *P. aeruginosa*, *K. pneumoniae* and yeasts are most commonly isolated from mares which had a previous history of intrauterine antibiosis or compromised uterine immune defence mechanisms.

It is thought that in some of the mares with delayed uterine clearance, defective uterine immune defence mechanisms may contribute to persistence of infection (Watson, 1987, 1988c). Neutrophils entering the uterine lumen are the first line of immune defence against invading bacteria. Their migration from the blood is enhanced by chemotatic factors present in uterine fluid (Blue et al., 1984; Watson, 1988a) which are increased after introduction of infection and inflammation into the uterus (Watson et al., 1987a,b, 1988a,b).

It has been suggested that uterine neutrophils collected from susceptible mares 12 h after infection show a premature migration dysfunction in chemotactic chambers (Liu et

al., 1986). However, when the neutrophils were removed from uterine factors, uterine neutrophil migration was found to be similar in resistant and susceptible mares (Troedsson et al., 1993d). In another study, uterine-derived neutrophils were not capable of migrating under agarose, possibly because neutrophils which have phagocytosed bacteria have run out of plasma membrane for locomotion (Watson et al., 1987a). However, the importance of neutrophil migration once the cells have reached the site of inflammation is questionable (Wilkinson, 1982). No defect in migration of blood-derived neutrophils has been found in susceptible mares (Watson et al., 1988; Troedsson et al., 1993d).

Once neutrophils have reached the uterine lumen their capacity to ingest and kill bacteria is critical in elimination of infection. The phagocytic ability of uterine neutrophils from susceptible mares is less than that from resistant mares (Cheung et al., 1985; Watson et al., 1987a; Troedsson et al., 1993d). However, if the uterine neutrophils were placed in an optimal environment, those collected from susceptible mares were shown to be fully functional (Troedsson et al., 1993d). It was concluded that factors in uterine secretions from susceptible mares interfered with phagocytosis. It has previously been reported that uterine secretions from susceptible mares were significantly worse at promoting phagocytosis than secretions from resistant mares (Watson et al., 1987a). Opsonisation by uterine secretions is dependent on both complement and specific antibody (Brown et al., 1985; Hansen and Asbury, 1987; Watson, 1988b; Hakansson et al., 1993), and a deficiency in complement was suggested by Asbury et al. (1984) which initiated the use of intra-uterine plasma therapy (Asbury, 1984). However, later work showed that haemolytic complement activity was high in flushings from susceptible mares presumably because of persistent uterine inflammation (Watson et al., 1987a). More recent work has shown that although specific endometrial antibody titres to *S. zooepidemicus* are similar in resistant and susceptible mares, the opsonic activity of these antibodies is lower than those of resistant mares (Watson and Stokes, 1990; Le Blanc et al., 1991). The importance of antibody in elimination of uterine bacterial infection has further been demonstrated by active (Widders et al., 1995) and passive (Watson and Stokes, 1988a) immunisation studies.

S. zooepidemicus also has the ability to physically adhere to endometrial epithelial cells (Watson et al., 1988) and a recent study has shown that bacterial adherence to epithelial cells is greater in mares with category III endometria (Ferreria-Dias et al., 1994). These results resemble the increased bacterial adherence to urinary tract epithelial cells reported to occur in women susceptible to recurrent urinary tract infections (Fowler and Stamey, 1977) and deserve further study.

The cellular immune system has not received much attention in the equine uterus. There appears to be no deficiency of T lymphocyte subsets $CD4^+$ or $CD8^+$ (Watson and Thomson, 1996) in susceptible mares. Uterine MHC II expression was significantly upregulated in mares with endometritis, and a high level of staining was seen in endometrial epithelial cells (Watson and Dixon, 1993). Macrophage function seems to be normal in susceptible mares (Watson and Stokes, 1988b) although local antigen uptake by endometrial macrophages was not studied. In fact, a recent study has shown that macrophage numbers do not increase as much as would be expected in the endometrium of susceptible mares (Summerfield and Watson, 1998) and may be responsible for a deficiency in antigen processing and handling at the uterine level.

6. Diagnosis of post-breeding endometritis

A detailed breeding history should be obtained. Mares should then be carefully evaluated prior to breeding to check that the perineal and vulvar anatomy is normal, the cervix opens in oestrus and closes in dioestrus, that no free intrauterine fluid is present on transrectal ultrasonography, and that endometrial smear and culture contain neither neutrophils nor a significant growth of known uterine pathogens.

Unfortunately, mares can present as reproductively normal at the start of the breeding season which gives the clinician no forewarning of the potential susceptibility of the mare. A diagnostic scintigraphic technique has been employed to identify mares with delayed uterine clearance (Le Blanc et al., 1994). However, scintigraphy is not readily available in many veterinary practices. Severe histopathological changes in an endometrial biopsy correlate well with susceptibility to PMIE (Troedsson et al., 1993a). However, biopsies are often not routinely collected from mares at the start of the breeding season and mild to moderate histopathological changes, into which the majority of mares fall, do not correlate well with susceptibility.

After breeding, a mare which retains intrauterine fluid for more than 12 h is considered to have PMIE (Troedsson, 1997a).

7. Treatment

The majority of mares with intrauterine fluid after breeding have negative culture results but have many neutrophils present in an endometrial smear. Treatment is generally aimed at assisting the uterus to physically clear contaminants and inflammatory products. Because of the association between semen in the uterus and PMIE, it is generally accepted that mares should be mated only once during oestrus. It is recommended to perform large volume lavage at 6 to 12 h after mating in susceptible mares (Troedsson et al., 1995; Knutti et al., 1997). Alternatively, a single injection of oxytocin has been used to facilitate uterine emptying (Allen, 1991; Pycock, 1994) 3 to 12 h after mating. In the latter study, oxytocin treatment was followed 30 min later by intrauterine infusion of antibiotics. This combined therapy was found to result in higher pregnancy rates than use of intravenous oxytocin or intrauterine antibiotics on their own (Pycock and Newcombe, 1996a). However, the population of mares in this study was not selected for susceptibility to endometritis, and a previous study showed that uterine lavage on its own was as effective as uterine antibiotics in reducing uterine inflammation (Troedsson et al., 1995). Troedsson (1997a) has also suggested that $PGF_{2\alpha}$ may be useful in treatment of susceptible mares. Ten milligrams of $PGF_{2\alpha}$ causes 5 h of increased myoelectrical activity, whereas 20 IU oxytocin causes only 1 h of increased activity. However, in a recent study, Combs et al. (1996) showed that oxytocin was more effective than $PGF_{2\alpha}$ at clearing radiocolloid from the uterus within 30–60 min after inoculation. Dose of oxytocin may be important in effective treatment. Uterine activity increases with intravenous doses of oxytocin from 2.5 to 10 IU and changes to intrauterine pressure are stimulated within 30 to 65 s, with peak response accuring in the first 5 to 10 min (Cadario et al., 1999). Interestingly, the first pressure wave was greater

than the succeeding waves and probably evacuated the majority of the uterine contents. Recent work has shown that pregnancy rates decrease when mares are treated with 25 IU compared with 15 IU oxytocin after breeding (Rasch et al., 1996), probably due to induction of tetanic spasm of the myometrium by the higher dose. Some clinicians are now using repeated doses of oxytocin throughout the day to ensure uterine clearance. However there is some evidence that the oxytocin has a reduced effect on uterine contractile activity in the second and subsequent treatments when given at intervals of 2.5 h (Nikolakopoulos, 1998). Transient uterine refractoriness after repeated oxytocin administration has also been reported in ewes (Sheldrick and Flint, 1986). A combination of lavage and oxytocin therapy is now the preferred method of treatment in many centres. It is now thought that antibiotics may not be necessary, even in cases of bacterial contamination, if mares are treated by large volume lavage and/or ecbolic agents within 12 h of mating (Troedsson, 1997a; Nikolakopoulos and Watson, 1999). The indiscriminate use of intrauterine antibiotics administered to all mares cannot be justified and should be discouraged.

8. Conclusion

Post-breeding endometritis is still the subject of much research. Major challenges which now face researchers include development of practical ways of determining susceptibility to PMIE prior to breeding which are easily accessible to the veterinary practitioner, and identification of the precise cause of the defect in uterine contractility in mares with PMIE.

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Concentrations of uterine luminal prostaglandins in mares with acute and persistent endometritis

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Summary

Intrauterine infusion of 1 per cent oyster glycogen solution was used to induce acute endometritis in four genitally normal mares. Numbers of viable neutrophils recovered in uterine washings had increased by 1 h after infusion and remained elevated for at least 72 h. There was a significant correlation between numbers of viable neutrophils and total protein concentrations and between prostaglandin (PG)F and PGE_2 concentrations in washings. There was also a significant relationship between concentrations of 15-keto-13, 14-dihydro $PGF_{2\alpha}$ in plasma and PGF in washings. Intrauterine concentrations of PGF were influenced by cycle stage and in turn the induced acute endometritis interfered with normal ovarian function. Mares with persistent endometritis had significantly higher concentrations of PGF and total protein and percentage of neutrophils and monuclear cells in washings than normal mares. White blood cells from mares were capable of producing PGF and PGE_2 *in vitro.*

Introduction

A MAJOR source of infertility in the mare is recurrent and persistent endometritis (Peterson, McFeely and David 1969; Hughes and Loy 1975). At coitus, the stallion ejaculates semen containing bacteria and other environmental contaminants through the mare's open cervix into the uterine lumen. All mares are thus infected by natural mating. In most mares, local uterine defence mechanisms rapidly eliminate bacteria and resolve the associated transient acute endometritis within 72 h (Peterson *et al* 1969). Other, usually older, multiparous mares appear to be unable to eliminate infection introduced at coitus and the associated endometritis persists.

In addition to signs of chronic inflammation (mononuclear cell infiltration, glandular degenerative changes and stromal fibrosis), endometrial biopsy specimens taken from these susceptible mares show signs of acute endometritis (polymorphonuclear cell infiltrations) and this type of endometrial pathology is termed persistent endometritis (Ricketts 1978). The difference between these resistant and susceptible mares may relate more to as yet undefined 'mare factors' controlling uterine defence than to primary invasion by bacteria (Blue and Mannwacker 1984).

It is important to understand the pathogenesis of acute endometritis in normal mares to aid investigations of mares which develop persistent endometritis. Prostaglandins, especially prostaglandin (PG) E_2, are known to be important mediators in other sites of inflammation, potentiating vasodilation and increased vascular permeability associated with the influx of serum proteins and white blood cells to sites of inflammation (Weissmann 1980). Measurement of the concentrations of prostaglandins in the medium of *in vitro* cultures of endometrium and in blood vessels draining the uterus have shown that the endometrium synthesises prostaglandins with the main products of arachidonate metabolism being $PGF_{2\alpha}$ and PGE_2 (Barcikowski, Carlson, Wilson and McCracken 1974; Downie, Poyser and Wunderlich 1974; Abel and Kelly 1979).

These two prostaglandins have an important role, although that of PGE_2 has not yet been fully defined, in regulating the oestrous cycle and in the establishment of pregnancy. Disturbance of the normal pattern of prostaglandin synthesis within the uterus has been measured in plasma by a metabolite of $PGF_{2\alpha}$, 15-keto-13, 14-dihydro $PGF_{2\alpha}$ (PGFM) and has shown that plasma concentrations of PGFM may be elevated in the presence of uterine infection (Neely *et al* 1979).

The present investigation employed sequential uterine washings in mares with experimentally induced endometritis to study luminal concentrations of PGF and PGE_2 along with total protein concentrations and neutrophil numbers. Concentrations of progesterone, oestradiol-17ß and PGFM were measured in plasma to monitor any changes in the oestrous cycle.

Materials and methods

Animals

Thirteen mares aged between four and 20 years and weighing from 250 kg to greater than 500 kg were used. All mares had a palpably normal genital tract with no histological evidence of endometritis in endometrial biopsies by the criteria of Kenney (1978). There was no bacterial growth from endometrial swabs incubated on blood agar plates for 48 h at 37°C.

A further 16 mares (14 mares heavier than 500 kg and two mares lighter than 500 kg) were identified as having a history typical of persistent endometritis. These mares had experienced repeated conception failure and/or foetal death and microorganisms were frequently recovered from uterine swabs. Endometrial smears stained with Leishman's showed the presence of neutrophils and mononuclear cells. Endometrial biopsies showed evidence of chronic glandular degenerative changes with associated periglandular fibrosis, diffuse

mononuclear cell infiltration of the stroma and a mild to severe diffuse infiltration of neutrophils.

Sampling

Blood samples (10 ml) were collected from the jugular vein into heparinised or EDTA containing vacutainer tubes (Becton-Dickinson).

After cleansing of the vulva and perineal region uterine washings were performed as described by Mitchell *et al* (1982) using 40 ml sterile phosphate buffered saline (PBS; pH 7.0) for mares weighing less than 500 kg and 80 ml PBS for mares more than 500 kg. The washings were kept on ice until centrifuged at 125 *g* for 15 mins at 4°C. The leucocytes in the sediment were resuspended in 1 ml PBS and counted using a haemocytometer. Viability was assessed by exclusion of trypan blue dye (0.1 per cent, 1:1 v/v). The supernatant was centrifuged at 10,000 *g* for 30 mins at 4°C and was stored in aliquots at −70°C.

Plasma progesterone assay

Plasma was assayed by the method described by Watson and MacDonald (1984) for cattle, with minor modifications. Analar water (1 ml; BDH) was added to 0.1 ml plasma before extraction with 2 × 5 ml light petroleum ether. All samples were included in one assay. Sensitivity of the assay with reference to the standard curve was 25 pg. Recovery of radiolabelled progesterone was 95 per cent and the within-assay coefficient of variation for replicate spiked samples was 16 per cent for 50 pg and 8 per cent for 400 pg progesterone.

Plasma oestradiol-17ß assay

The method used was a modification of the technique described by Dobson and Dean (1974). The antiserum (5322) was provided by Roussel-Uclaf, Paris and was raised against 17ß-oestradiol-7-carboxymethyloxime-BSA and cross-reacted with oestradiol-17ß 100 per cent, oestriol 0.6 per cent, oestrone 0.4 per cent, oestradiol-17α 0.3 per cent and other steroids tested less than 0.1 per cent. The final dilution of antiserum used in the assay was 1: 1×10^5. Recovery of radiolabelled oestradiol [2, 4, 6, 7-^{3}H) oestradiol:85-110 Ci/mmol; Amersham] following a double extraction in diethyl ether was 79 per cent. The within-assay coefficient of variation in spiked plasma was 10 per cent at 5 pg and 6 per cent at 50 pg oestradiol. Sensitivity with respect to the standard curve was 2.5 pg. All samples were included in one assay.

Plasma PGFM assay

The method used was as described by Watson (1984). Main cross-reactivities were with 15-keto-$PGF_{2\alpha}$ 16 per cent, 13,14-dihydro $PGF_{2\alpha}$ 4 per cent, $PGF_{2\alpha}$ 0.4 per cent and 15-keto-13, 14-dihydro PGE_2 1.7 per cent. Assay sensitivity with reference to the standard curve was 5 pg. Recovery of radiolabelled PGFM after a double extraction with diethyl ether was 75 per cent. The within-assay coefficient of variation in spiked plasma was 14 per cent at 100 pg and 6 per cent at 200 pg PGFM. All samples were included in one assay.

PGF and PGE_2 assays

Concentrations were determined by radioimmunoassay of unextracted uterine washings. Standard solutions of $PGF_{2\alpha}$ (12.5, 25, 50, 100, 200, 400, 800, 1600 pg/0.1 ml) and of PGE_2 (1.25, 2.5, 5, 12.5, 25, 50, 100, 200 pg/0.1 ml) were prepared in ethanol and stored at 4°C (due to the stability of $PGF_{2\alpha}$ at this temperature) and −70°C, respectively. Standards (0.1 ml) were dried down in siliconised glass tubes at 45°C under a stream of nitrogen gas prior to assay.

Antisera were raised in rabbits against BSA-conjugates for both hormones. The main cross-reactivities (more than 0.5 per cent) of the anti-PGF were $PGF_{1\alpha}$ 75 per cent and $PGF_{2\beta}$ 1.5 per cent and of the anti-PGE_2 was PGE_1 3.2 per cent. The antisera were added to uterine washings (0.1 ml) or to 0.1 ml PBS (PBS, 0.1 per cent gelatine and 0.4 g sodium azide) in tubes containing standards. [5, 6, 8, 9, 11, 12, 14, 15 (n)- ^{3}H] − $PGF_{2\alpha}$ (160-180 Ci/mmol) or -PGE_2 (140-170 Ci/mol: Amersham) was added to all tubes (4000 counts per min/0.1 ml) and incubated overnight at 4°C.

Antibody-bound hormone was separated from free hormone by a charcoal separation procedure using 1 ml of 0.25 per cent charcoal (Norit-A; Sigma) with 0.025 per cent dextran suspended in Analar water. After incubation at 4°C for 10 mins, the tubes were spun at 2000 *g* for 10 mins at 4°C and the supernatant was decanted into glass scintillation vials. Cocktail-T scintillant (10 ml; BDH) was added to each vial and radioactivity counted in a liquid scintillation counter (Searle Isocap 300). Concentrations of prostaglandins were calculated by extrapolation on to a standard curve. Because of the cross-reactivity of the anti-$PGF_{2\alpha}$ with $PGF_{1\alpha}$ results are expressed as immunoreactive PGF.

Approximately 30 per cent of the radioactive prostaglandin was bound to antibody at a final dilution of 1:300 ($PGF_{2\alpha}$) and 1:30 (PGE_2) in the absence of unlabelled hormone. Standard curves prepared using assay buffer and uterine washings showed good parallelism. The coefficient of correlation for amount added: amount measured was 0.999 for $PGF_{2\alpha}$ and 0.977 for PGE_2. The within-assay coefficient of variation for $PGF_{2\alpha}$ was 11 per cent over the range 50 to 500 pg and for PGE_2 was 10 per cent over the range 12.5 to 50 pg. Sensitivity with respect to the standard curve was 12.5 pg for $PGF_{2\alpha}$ and 1.25 pg for PGE_2. All samples were included in one assay.

Total protein assay

Total protein was determined in uterine washings using a Microprotein Rapid Stat Diagnostic Kit (Sherwood Medicals) based on formation of a dye-protein complex with Coomassie brilliant blue dye.

EXPERIMENTAL DESIGN

Experiment 1

The response of the uterus to infusion with glycogen was determined in six pony mares divided into three groups. Uterine washings (40 ml) were collected immediately before infusion of either sterile PBS (pH 7.0), or 0.1 per cent or 1.0 per cent oyster glycogen (Sigma Chemicals) dissolved in sterile PBS (50 ml). Washings were collected from one mare in each pair at 6 h and from the other at 16 h after infusion.

Experiment 2

Four of the genitally normal pony mares from Experiment 1 were used. Two of the mares were in mid-dioestrus, one was in oestrus and one was at luteolysis. On the first day of the experiment (Day 0) uterine washings were collected immediately before infusion of 1 per cent oyster glycogen. Uterine washings were then performed at 1, 3, 6, 24, 48, 72 and 144 h after infusion. Washings were examined for number of live neutrophils. Concentration of total protein, PGE_2 and PGF were measured and expressed as total concentrations by multiplying

y volume recovered. Washings were streaked on to blood agar lates and incubated at 37°C for 48 h.

Blood samples were collected from the jugular vein into eparinised vacutainer tubes twice in the week before infusion f glycogen, immediately before collection of each uterine ashing and on Days 8 and 10 after infusion. Blood was spun at 000 *g* for 10 mins at 4°C and the plasma frozen in aliquots at -20°C. Plasma samples were assayed for concentrations of rogesterone and oestradiol-17ß and PGFM was measured in amples collected before each uterine washing.

xperiment 3

Uterine washings were collected on one occasion from the 16 ares with persistent endometritis and 13 genitally normal ares (six mares more than 500 kg, seven mares less than 00 kg). None of these mares was sampled at luteolysis. The ercentage of neutrophils and mononuclear cells was etermined in uterine washings and concentrations of PGF, GE_2 and total protein were measured.

n vitro *synthesis of prostaglandins*

The ability of white blood cells to synthesise prostaglandins as determined by stimulating phagocytosis of live bacteria. lood was collected into vacutainers containing EDTA and d blood cells were allowed to sediment by standing at room mperature for 30 mins. The leucocyte-rich supernatant was ollected and spun at 2000 *g* for 5 mins at 4°C. The super-atant was discarded and the white cells resuspended in 1 ml anks balanced salt solution containing 20 mM HEPES (HBSS, H 7.2: Flow Laboratories). Remaining red cells were lysed by ixing the cells for 30 secs with 4.5 ml sterile distilled water. o regain isotonicity, 0.5 ml of 10 × HBSS was added. The lls were washed three times in PBS before being resuspended 10^7 neutrophils/ml HBSS. More than 95 per cent of cells ere viable after this procedure.

White blood cells (0.2 ml) were incubated at 37°C with 1 per nt serum from a gelding (0.3 ml) and either with or without e *Streptococcus zooepidemicus* (0.1 ml, 3 × 10^6/ml) in plicate 2.5 ml plastic stoppered vials on a coulter roller. In bes without bacteria, 0.1 ml of PBS was substituted. uplicate tubes were included which had 0.2 ml HBSS bstituted for white blood cells. After 2 h the tubes were spun 2000 *g* for 15 mins at 4°C and the supernatant was decanted d stored in aliquots at −70°C until assay.

atistical analyses

Results were analysed using Pearson's correlation efficient and the Student's *t* test. Values of $P<0.05$ were nsidered to be significant.

ABLE 1: Number of viable neutrophils recovered from uterine ashings of six mares after infusion of phosphate buffered line or oyster glycogen

eatment	Number of viable neutrophils		
	Pre-infusion	6 h post infusion	16 h post infusion
hosphate buffered saline	0	1.0×10^7	1.0×10^7
1% oyster glycogen	0	6.4×10^6	1.0×10^6
% oyster glycogen	0	1.0×10^8	1.0×10^8

Results

Experiment 1

All treatments resulted in recovery of a large number of viable neutrophils from the uterine lumen (Table 1). Oyster glycogen at a concentration of 1 per cent produced at least 10 to 100 times more neutrophils at 6 and 16 h after infusion than PBS or 0.1 per cent oyster glycogen. There was no bacterial growth from any of the washings incubated on blood agar plates for 48 h at 37°C.

Experiment 2

The mean ± sem volume of uterine washings recovered was 37.5 ± 0.79 ml. Numbers of live neutrophils in washings had increased by 1 h after infusion (Fig 1). In all mares numbers of neutrophils remained elevated until at least 72 h after infusion although there was a large difference in magnitude of response between mares. Bacteria (ß-haemolytic streptococci) were isolated on one occasion from the washings of **Mares 2** (24 h) and **4** (72 h) and *E coli* were isolated from the washings of **Mare 3** (24 h). Each isolation was associated with recovery of a large number of live neutrophils. There was a highly significant correlation ($P<0.001$) between numbers of live neutrophils and total protein in uterine washings ($r = 0.62$). Concentration of total protein rose from 1.5 ± 0.29 mg before infusion to 19.3 ± 8.30 mg by 24 h after infusion.

Concentrations of PGF and PGE_2 in washings were very low before infusion and rose between 1 and 6 h after infusion (Fig 2). Magnitude and pattern of response varied greatly between mares but, in general, concentrations decreased to low levels at 24 h followed by a secondary rise lasting a variable period of time. Concentrations of PGF and PGE_2 were highly correlated ($r = 0.79$, $P < 0.001$) and plasma

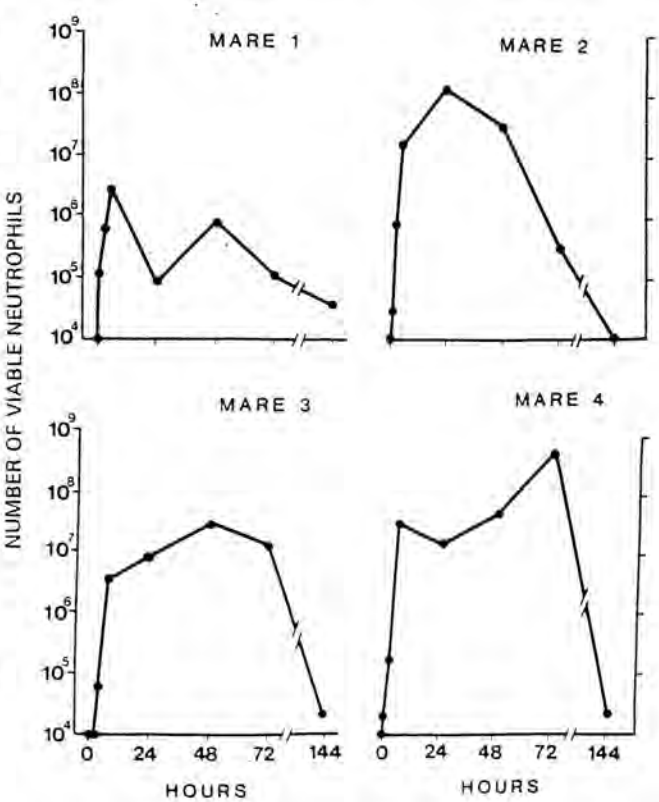

Fig 1: Number of viable neutrophils in uterine washings from mares after intrauterine infusion of oyster glycogen

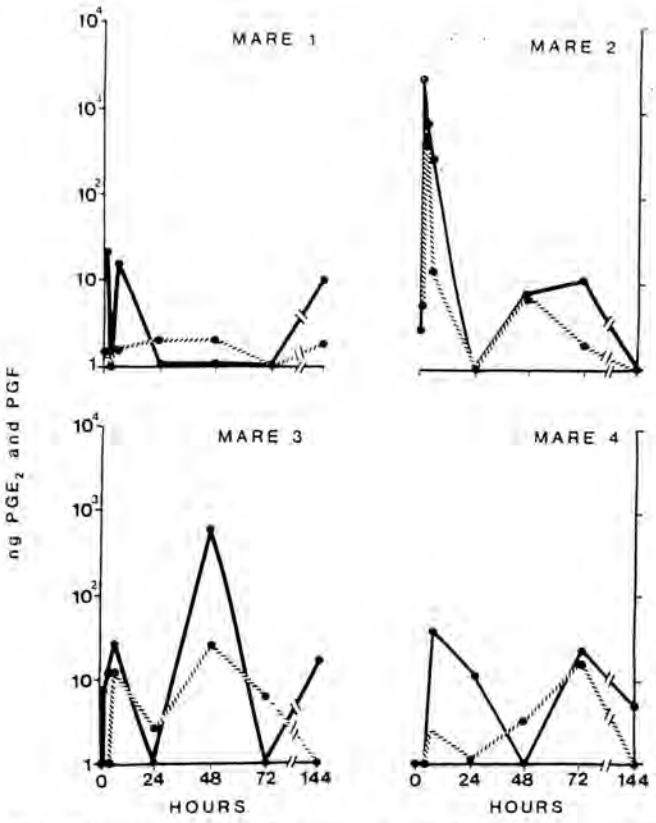

Fig 2. Concentrations of PGF (●——●) and PGE_2 (●·····●) in uterine washings from mares after intrauterine infusion of oyster glycogen

concentrations of PGFM were significantly correlated with PGF concentrations in uterine washings ($r = 0.37$, $P < 0.05$) but not with PGE_2 ($r = 0.14$). Concentrations of PGF and PGE_2 in washings were not significantly correlated either with numbers of live neutrophils or concentration of total protein.

At infusion (Day 0) two mares (3 and 4) were in mid-dioestrus (Fig 3); by Day 2 progesterone concentrations were decreasing and reached very low levels by Day 6. Plasma concentrations of PGFM increased sharply on Days 1 to 4. These hormone profiles demonstrate luteolysis after infusion and a premature return to oestrus with increasing concentrations of oestradiol in plasma by Day 10.

Mare 1 was in oestrus on Day 0 (Fig 3). Plasma concentrations of PGFM showed a small peak on Day 1 and rose again on Day 6. Progesterone concentrations were elevated by Day 6 but by Day 10 the mare was again coming into oestrus with rising plasma concentrations of oestradiol.

Mare 2 was undergoing luteolysis on Day 0 and progesterone concentrations remained low until Day 10. This was the only mare with elevated concentrations of PGFM before infusion and in this mare there was a rise to higher concentrations on Day 2 after infusion.

Experiment 3

The mean ± sem volume of uterine washings recovered from mares greater than 500 kg was 58.8 ± 4.41 ml and from mares less than 500 kg it was 37.1 ± 1.35 ml. There was no significant difference between genitally normal mares and mares with persistent endometritis in volume of washing recovered. Beta-haemolytic streptococci were isolated from three of the mares with persistent endometritis. None of the uterine washings from the other mares produced bacterial growth on blood agar plates after a 48 h incubation at 37°C. Concentrations of PGF, PGE_2 and total protein and percentage of neutrophils and mononuclear cells were significantly higher in uterine washings from mares with persistent endometritis than in normal mares (Table 2). Uterine washings from mares greater than 500 kg contained significantly ($P<0.01$) more mononuclear cells (10.1 ± 2.56 per cent) than mares less than 500 kg (0.3 ± 0.29 per cent). However, none of the other measurements was significantly different between the two groups of normal mares.

In vitro *synthesis of prostaglandins*

Table 3 shows that equine white blood cells are capable of synthesising significant concentrations of PGF and PGE_2. In the absence of bacteria, white blood cells produced lower concentrations of PGF and PGE_2. No detectable concentrations of prostaglandins were measured in tubes which did not contain white blood cells.

Discussion

Oyster glycogen has been used as an irritant in various species to produce an immunologically non-specific inflammatory response in the peritoneal cavity (Hirsch 1956), mammary gland (Paape, Pearson, Wergin and Guidry 1977) and uterus (Watson 1985). In the present study intrauterine infusion of either PBS or oyster glycogen caused a massive increase in the number of live neutrophils recovered from uterine washings by 6 h after infusion. At a concentration of 1 per cent, infusion of oyster glycogen resulted in collection of adequate numbers of live neutrophils for use in tests of neutrophil function and provides an alternative in mares to the more standard infusion of endotoxin or live bacteria into the uterus.

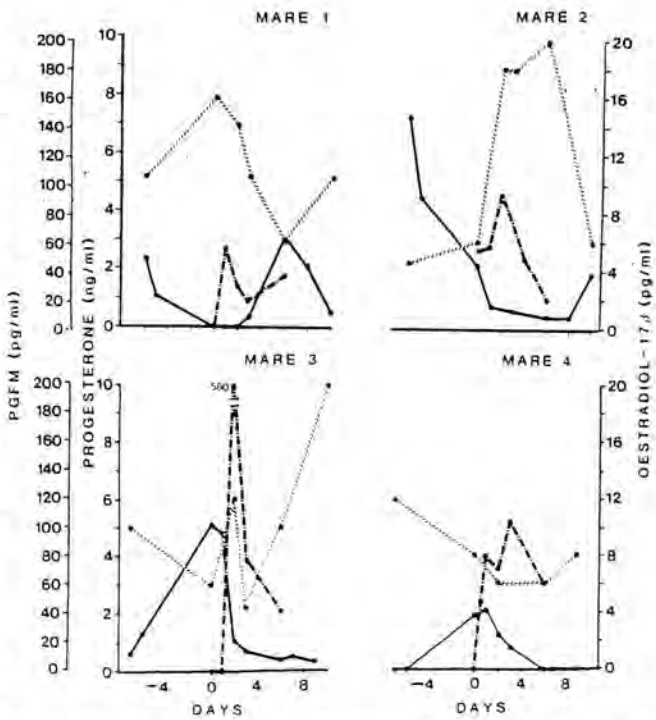

Fig 3: Peripheral plasma concentrations of PGFM (●▬▬●) oestradiol-17β (●·····●) and progesterone (●——●) in mares before and after intrauterine infusion of oyster glycogen (Day 0)

ABLE 2: Comparison of components of uterine washings in ares with and without persistent endometritis

	Normal mares (n = 13)	Mares with persistent endometritis (n = 16)	Level of significance
ɔnonuclear cells (%)	4.9 ± 1.82*	21.0 ± 5.13	$P < 0.01$
utrophils (%)	9.1 ± 4.23	56.8 ± 9.14	$P < 0.001$
tal protein (mg)	6.7 ± 1.89	124.4 ± 27.05	$P < 0.001$
GF (ng)	24.0 ± 12.17	141.8 ± 37.88	$P < 0.001$
GE_2(ng)	2.3 ± 0.95	6.5 ± 1.97	$P < 0.05$

Mean ± sem

In mares, endometritis has been reported to cause premature eolysis (Hughes and Loy 1975). A more recent study has own prematurely elevated concentrations of PGFM in asma which have been associated with luteolysis in two ares with endometritis (Neely *et al* 1979) and it was hypo-esised that uterine infection led to increased synthesis of $GF_{2\alpha}$ by the inflammatory process and/or the presence of cterial toxins. Pyometrial fluid from bitches, cows and rrets has been shown to contain high concentrations of $GF_{2\alpha}$ and PGE_2 (Heap and Poyser 1975) and in cattle the ncentrations of PGF in pyometrial fluid were significantly rrelated with plasma concentrations of PGFM (Nkuuhe 82).

In mares, metabolism of $PGF_{2\alpha}$ is different from that in the w (Kindahl *et al* 1984) and it was not known how accurately easurement of PGFM concentrations in blood reflected trauterine response to inflammation by production of ostaglandins. In the present study, with the exception of GF concentrations in **Mare 1,** all mares showed an elevation intrauterine PGF and PGE_2 concentrations within 1 to 6 h infusion of glycogen. In these mares there was a significant rrelation between concentrations of PGF in uterine shings and of PGFM in plasma illustrating that in mares th acute endometritis, plasma concentrations of PGFM are uitable indicator of uterine PGF synthesis.

It is known that cervical dilation can induce luteolysis urtgen and Ganjam 1979). Prolonged vaginal and cervical nipulation and infusion of large volumes of saline (500 ml) n stimulate prostaglandin synthesis (Neely *et al* 1979; tteridge, Renard and Goff 1985) as measured by plasma ncentrations of PGFM. Furthermore, uterine luminal con-ntrations of PGF have been elevated after a protracted pro-dure for recovery of uterine washings which involved 5 mins uterine massage per rectum (Berglund, Sharp, Vernon and atcher 1982).

Other workers, using a washing procedure similar to that in e present study found that the length of dioestrus was un-fected when repeated uterine washings were performed in ccessive oestrous cycles (Strzemienski and Kenney 1984). In e present study it seems unlikely that the collection technique ntributed significantly to prostaglandin concentrations and premature return to oestrus because of the small volumes infused and the speed of the collection procedure in which there was no manipulation of the uterus per rectum. Although the procedure did involve dilation of the cervix. Furthermore, measurement of plasma PGFM concentrations at 5 min intervals from the start of the uterine washing procedure has shown that, except at luteolysis, PGFM concentrations return to pre-infusion levels within 30 mins and remain there for the next 24 to 48 h (E. D. Watson, unpublished data).

Uterine inflammation led to interference with normal ovarian cyclicity resulting in premature luteolysis in two mares which were in dioestrus at infusion and premature termination of the subsequent luteal phase in a mare which was infused during oestrus. The pattern of uterine prostaglandins in the mares in dioestrus was similar to the pattern of plasma PGFM described by Neely *et al* (1979) after infusion of large volumes of saline with an immediate response which subsided, followed by secondary elevations and luteolysis one to two days later.

The concentrations of PGF and PGE_2 measured in uterine washings were produced presumably by the endometrium and luminal and tissue white blood cells. Equine white blood cells produced prostaglandins *in vitro* and, as with human white blood cells, higher concentrations of prostaglandins were produced by stimulating phagocytosis (Weissmann 1980). It is likely that neutrophils and monocytes were the major contributors as lymphocytes have been reported to produce little or none in other species (Lewis 1983). A recent study with bovine white blood cells, however, showed that neutrophils and lymphocytes produced prostaglandins in equal amounts (Nkuuhe 1982).

The stage of oestrous cycle at infusion probably influenced the quantity of prostaglandins synthesised by the endometrium resulting in a massive response in the mare which was infused during luteolysis. It has been shown in mares that decreasing concentrations of progesterone after sustained exposure to high levels, followed by high concentrations of oestradiol are necessary for maximal production of $PGF_{2\alpha}$ (Vernon, Zavy, Asquith and Sharp 1981). This pattern of blood hormones was present in **Mare 2.** In contrast, the mare which was in oestrus at infusion failed to show elevated concentrations of PGF after infusion. It is not known how, in the mare, intrauterine concentrations of PGE_2 are influenced by stage of cycle but in ewes PGE_2 is thought to be luteotrophic (Speroff and Ramwell 1970; Henderson, Scaramuzzi and Baird 1977).

The superimposition of effects of reproductive hormones on prostaglandin synthesis probably explains the lack of relationship between prostaglandins and number of neutrophils recovered from washings. There was a close relationship, however, between the total number of live neutrophils and concentrations of total protein as would be expected at sites of inflammation (Weissmann 1980) caused by vasodilation, increased permeability of blood vessels and release of chemotactic substances attractive to neutrophils. The first few hours after invasion by bacteria are very important in establishment and subsequent course of infection. All mares showed a large response by 6 h after infusion but the two mares with high plasma concentrations of oestradiol had substantially more luminal neutrophils by 3 h than the two mares in dioestrus. Increased speed of mobilisation of neutrophils in the presence of high concentrations of plasma oestradiol has been reported in rabbits (Hawk 1958) and ewes (Hawk, Turner and Sykes 1961) and might help to explain increased resistance of the uterus to infection during oestrus.

As protein and prostaglandin concentrations were not significantly different between normal mares which were greater than or less than 500 kg it was considered valid to include them in one group and compare them with persistently

BLE 3: The ability of white blood cells* from four mares to oduce prostaglandins *in vitro* after 2 h at 37°C

	Prostaglandins (ng/10^7 neutrophils)	
	Without *S zooepidemicus* (mean ± sem)	With *S zooepidemicus* (3×10^5) (mean ± sem)
F	0.97 ± 0.03	1.54 ± 0.54
E_2	0.19 ± 0.05	1.50 ± 0.62

0.3 ± 7.2 per cent neutrophils

endometritic mares most of which were greater than 500 kg. In the mares with persistent endometritis, concentrations of total protein were elevated as reported by Williamson, Dunning, O'Connor and Penhale (1983) and there was a higher percentage of luminal neutrophils to other cell types, a property which has been employed clinically in diagnosis of endometritis using endometrial smears (Wingfield Digby 1978). There was also a significant difference between the two groups in percentage of mononuclear cells, probably due to the large stromal population in mares with persistent endometritis.

Prostaglandins E_2 and F were significantly higher in mares with persistent endometritis. Prostaglandins of the E and F series can inhibit humoral immune responses such as B cell activation and antibody production (Goodwin and Webb 1980). Therefore, the presence of elevated intrauterine concentrations of prostaglandins in mares with persistent endometritis which occurred even in the absence of infection, may play an important part in reducing the resistance of these mares to chronic intrauterine infection after bacterial challenge.

There was no significant difference in mean (± sd) plasma concentrations of PGFM in genitally normal mares (26.9 ± 13.60 pg/ml) and in mares with persistent endometritis (33.9 ± 12.70 pg/ml). Thus it would seem that the high intrauterine concentrations of PGF were not absorbed in mares with persistent endometritis and explains the apparent lack of interference with normal cyclicity in mares with this condition.

Uterine inflammation in the mare seems to resemble inflammation at other sites and in other species with the added complication in mares with experimentally-induced acute endometritis, of modification of inflammatory response with stage of cycle and in turn interference with normal ovarian cyclicity by the inflammatory response.

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ABSTRACTS

urgery

st anesthetic hemorrhagic myelopathy in a horse

VICH, J. V., LeCOUTEUR, R. A., STASHAK, T. S., GRANDY, L. and WHALEN, L. R. (1986) *J. Am. vet. med. Ass.* **188**, 0-301.

)ST anaesthetic haemorrhagic myelopathy has been orted from Switzerland, England and Canada. This paper cribes a similar case in the USA.

The report concerned a one-year-old Quarterhorse colt dergoing surgery for castration. Xylazine, guaiphenesin and amine induction was followed by maintenance with halone. The horse was in dorsal recumbency. Anaesthesia was parently uneventful and lasted 45 mins.

Neurological dysfunction was apparent as soon as the horse d to stand. It was able to use the forelimbs, but the hindbs remained rigidly extended and no pain reflex could be ited. A lesion affecting the full thickness of the spinal cord ween the brachial and lumbosacral enlargements was pected. The horse's condition did not improve during the owing 16 h and euthanasia was performed.

Gross post mortem examination and histology demonated acute haemorrhagic myelopathy between CE6 and T8. emorrhage was most severe in the grey matter, especially in dorsal horns. Early neuronal degeneration was seen.

The authors discuss the possible causes of this condition n and consider that the following may be contributory tors. A young, rapidly growing animal where the spinal culature may not be fully developed; dorsal recumbency ere positioning and the weight of abdominal viscera may mpromise blood supply to, and particularly the venous rn from the spine; halothane anaesthesia which is otensive. They suggest that hypotension should be avoided his may potentiate the problem.

stractor's comment. — This is a rare but disastrous plication of general anaesthesia in the horse. Aetiology is ely speculative at present and more detailed knowledge of anatomy and function of spinal vasculature is urgently uired in order to provide a rational approach to prevention. This condition must be regarded as a potential, if rare, ard of general anaesthesia where the horse is in dorsal mbency, particularly in the young animal. Perfusion of spine is less likely to be compromised if halothane induced cardiovascular depression is kept to a minimum by maintaining as light a plane of anaesthesia as possible. It may be beneficial to tilt the horse slightly, so that vasculature is not compromised symmetrically and so that pressure on the vena cava is reduced.

POLLY M. TAYLOR

Locomotor system and diseases

Slab fractures of the fourth and intermediate carpal bone in five horses

AUER, J. A., WATKINS, J. P., WHITE, N. A., TAYLOR, T. S. and ROONEY, J. R. (1986) *J. Am. vet. med. Ass.* **188**, 595-601.

FIVE horses were presented with lameness of 10 to 28 days duration. All had sagittal or comminuted fractures of the fourth carpal bone and three also had a frontal plane fracture of the intermediate carpal. The delay in diagnosis and treatment was thought to be due to the mild degree of lameness. Three cases had acquired a valgus deformity of the knee secondary to fracture. All horses underwent surgical reduction, the screws transfixing two adjacent carpal bones in four of the cases. For immediate postoperative recovery the limb was bandaged heavily and a splint applied.

All cases developed degenerative joint disease and osteophyte formation which restricted carpal flexion and prevented the horses racing. Three cases acquired a flexural deformity of the fetlock on the injured leg, probably consequent on prolonged reduced weightbearing. Four retired to stud and one case was destroyed following breakdown of the repair due to excessive exercise too soon after surgery. The mechanics and pathogenesis of the fractures are discussed.

Abstractor's comments. — These cases are interesting in that the fractures cause relatively little lameness, but can destabilise the carpus and unless treated soon after occurrence hold a poor prognosis for return to athletic activity.

D. R. ELLIS

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CELLULAR AND HUMORAL DEFENCE MECHANISMS IN MARES SUSCEPTIBLE AND RESISTANT TO PERSISTENT ENDOMETRITIS

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ABSTRACT

Watson, E.D., Stokes, C.R. and Bourne, F.J., 1987. Cellular and humoral defence mechanisms in mares susceptible and resistant to persistent endometritis. Vet. Immunol. Immunopathol., 16: 107–121.

Both random and directional migration of blood neutrophils from 9 mares susceptible to persistent endometritis were significantly less ($p < 0.05$) than neutrophils from 8 resistant mares. Serum from susceptible mares had significantly more ($p < 0.01$) chemotactic activity than serum from resistant mares. Although phagocytosis of yeast blastospores by blood neutrophils from 4 resistant and 3 susceptible mares was similar, uterine neutrophils from susceptible mares were significantly worse ($p < 0.01$) at phagocytosis than uterine neutrophils from resistant mares.

Uterine washings from 17 susceptible mares were significantly better at opsonising yeast blastospores than washings from 14 resistant mares; however, washings from both groups had a similar ability to promote killing of S. zooepidemicus by neutrophils. When an immunologically non-specific endometritis was induced, washings from 3 susceptible mares were significantly worse at promoting bactericidal activity by 144 h than washings from 4 resistant mares ($p < 0.01$). Haemolytic complement activity was significantly greater ($p < 0.001$) in washings from 17 susceptible mares than from 14 resistant mares. Induction of acute endometritis resulted in high levels of haemolytic complement activity in 2 of 3 susceptible mares at 24 and 144 h, but only in small increases in 4 resistant mares. Thus, some abnormalities in neutrophil function were detected and a possible defect in promotion of neutrophil bactericidal activity by uterine secretions from susceptible mares but there was no evidence for any deficiency in haemolytic complement activity.

INTRODUCTION

The persistence of intrauterine infection after introduction of bacteria by the stallion at coitus is recognised as an important cause of infertility in susceptible mares (Hughes and Loy, 1975) and leads to the development of persistent endometritis. The bacteria most frequently isolated from these mares are beta-haemolytic Streptococci (Millar and Francis, 1974). It is commonly suggested that susceptible mares have some deficiency in the local immune defence mechanisms of the uterus although the exact nature of this deficiency is not known (Peterson et al., 1969; Widders et al., 1984). The mucosal immune system of the mare's uterus differs from a number of other species in the increased dependence upon locally derived immunoglobulin

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(Widders et al, 1984). A number of studies have reported increased concentrations of immunoglobulins in uterine secretions from mares with persistent endometritis but have questioned their protective capacity (Asbury et al., 1980; Mitchell et al, 1982; Williamson et al., 1983).

It is well recognised that even in the presence of immunoglobulins, opsonisation of bacteria may be markedly enhanced by complement (Lachman, 1979), and deposition of C3b on bacterial cell walls has been shown to be necessary for efficient phagocytosis by equine neutrophils (Asbury et al., 1984). Haemolytic complement has not been detected in uterine secretions from mares even after induction of acute inflammation (Asbury et al, 1984) and its absence has been suggested to have a significant effect on the establishment of intrauterine infection.

Deficiencies in cellular defence mechanisms have been implicated in a small number of mares. Uterine-derived neutrophils from susceptible mares were significantly worse at phagocytosis and responding to chemotactic stimuli than uterine-derived neutrophils from resistant mares (Cheung et al., 1985; Liu et al., 1985).

The present study investigated phagocytic and migratory capacities of uterine and blood neutrophils from resistant and susceptible mares and the ability of uterine washings to promote both bactericidal activity of neutrophils and to facilitate deposition of C3b on yeast blastospores thereby enhancing phagocytosis. In addition, haemolytic complement activity was measured in uterine washings.

MATERIALS AND METHODS

Experimental mares and procedures

Seventeen mares which were between 7 and 20 years old were classified as susceptible to persistent endometritis from a history of recurrent uterine infection and subfertility. Confirmation was by histological examination of endometrial biopsies which showed degenerative and infiltrative chronic inflammatory changes as described by Ricketts (1978) with mononuclear cell infiltration and usually a superimposed neutrophilic infiltration from a recent acute infection. A further 14 mares aged between 4 and 20 years old were classified as resistant to uterine infection from their history and this was confirmed by rectal palpation and endometrial biopsy.

Uterine washings were collected from these mares and were used in bactericidal and yeast opsonisation assays and total haemolytic complement activity was measured.

Blood samples were collected from 8 of the resistant and 9 of the susceptible mares and leucocytes were isolated to study both random and

directional migration of their neutrophils under agarose.

Serum was collected from each of 9 resistant and 11 susceptible mares. The chemotactic activity of this serum was studied using neutrophils from a gelding.

Blood samples and uterine washings were collected 18 h after induction of inflammation by intrauterine infusion of S. zooepidemicus in 4 susceptible mares and 4 resistant mares during the winter anovulatory period. The phagocytosis of yeast blastospores by blood and uterine neutrophils was studied.

Acute uterine inflammation was induced during the winter anovulatory period in 3 susceptible mares and 4 resistant mares by intrauterine infusion of a sterile solution of 1% oyster glycogen (50 ml) and washings were collected prior to infusion and at 24 and 144 h after infusion. These washings were used in bactericidal assays and for measurement of total haemolytic complement activity.

Techniques

Blood neutrophil preparation

Blood samples were collected into Vacutainer tubes containing EDTA. Leucocytes were isolated by sedimentation and hypotonic lysis as described previously (Watson et al., 1986c). After washing the leucocytes three times in phosphate buffered saline (PBS: pH 7.3), the cell pellet was resuspended in Hank's Balanced Salt Solution (HBSS) containing 20 mM Hepes (pH 7.3; Flow Laboratories, Irvine). Leucocytes were counted in a haemocytometer and their viability assessed by exclusion of trypan blue dye. Neutrophils were differentiated by staining a cytospin preparation with Giemsa (Diff-Quick, American Hospital Supplies, Compton) and the neutrophil numbers were adjusted to 1×10^7/ml.

Uterine washing procedure

After cleansing the perineum and vulva, washings were collected via a Foley catheter (24 FG; 30 ml cuff) or an equine egg collection catheter (24 FG; 30 ml cuff; Franklin Medicals, High Wycombe) using 40 to 80 ml sterile PBS. Washings were kept on ice until further processing. A sample of each washing was streaked onto a blood agar plate and incubated at 37° C for 48 h.

Neutrophils were isolated by centrifugation at 125 x g for 10 min at 4° C and subsequently treated as described for blood neutrophils. Washings were further spun at 10,000 x g for 30 min at 4° C and stored at -70° C in aliquots.

Introduction of uterine infection

A genital strain of Streptococcus zooepidemicus was incubated overnight in Brain Heart Infusion Broth. The bacteria were washed twice in PBS, adjusted to 1 x 10^9 in 50 ml sterile PBS and infused into the uterus.

Neutrophil migration assay

Migration was studied using the agarose technique described previously (Watson et al, 1986a). Plates for random migration contained 10% heat-treated newborn calf serum in 1.15% agarose and plates for directional migration contained 0.2% bovine serum albumin in 0.75% agarose. Pooled horse serum stored at -70° C was used as the chemoattractant on directional migration plates. Five replicate plates were prepared for each horse. Neutrophils were allowed to migrate under agarose for 2.5 h at 37° C. After fixation with methanol and removal of the agarose, the neutrophils were stained with Leishman's stain. The distance migrated was calculated using an eyepiece graticule and the distance measured from the well to the last line of 10 squares which contained at least one neutrophil in each of five squares.

Chemotactic activity of serum

The neutrophil migration assay was performed using directional migration plates as described above using neutrophils from a gelding. Sera from 11 susceptible mares and 9 resistant mares were used as the chemoattractants.

Yeast phagocytosis assay

This was performed by a modification of the method described by Soothill and Harvey (1976). Plastic stoppered tubes were prepared in duplicate containing 0.2 ml Saccharomyces cerevisiae blastospores (1 x 10^8/ml), 0.1 ml pooled 1% horse serum and 0.1 ml neutrophils (1 x 10^7/ml). Tubes were included which contained PBS instead of uterine washing or serum. After incubation on a roller at 37° C for 10 min the tubes were spun at 50 x g for 5 min, the supernatant discarded and the cell pellet resuspended in 1.5 ml PBS. A cytospin preparation was stained with Giemsa and 100 neutrophils and their phagocytosed yeast blastospores were counted. Results were expressed as the percentage of neutrophils which had ingested $\geq$ 2 blastospores.

The effect of bactericidal activity on yeast phagocytosis by blood neutrophils was studied by incubating approximately 1 x 10^7 cfus/ml (0.1 ml) of S. zooepidemicus opsonised with 10% horse serum (0.1 ml) with blood neutrophils (0.1 ml; 1 x 10^7/ml) for 1.5 h at 37° C. The tubes (n = 6) were then centrifuged at 50 x g for 5 min and, after removal of the supernatant, the cells were washed twice with HBSS. The neutrophils were then resuspended

at 1 x 10^7/ml and used in the yeast phagocytosis assay as described above. The number of blastospores ingested per neutrophil was then compared with numbers ingested by neutrophils which had been incubated at 37° C in the absence of bacteria.

Yeast opsonisation assay

This assay was performed as described above with 0.1 ml uterine washing substituted for the horse serum and the incubation period extended to 45 min.

Bactericidal assay

A culture of a genital strain of Streptococcus zooepidemicus was grown up overnight in Brain Heart Infusion Broth at 37° C. The bacteria were washed twice in PBS and resuspended at 3.5 x 10^6/ml by calibration of optical density using a spectrophotometer. Duplicate tubes (A) were prepared containing 0.3 ml uterine washing, 0.2 ml neutrophils (1 x 10^7/ml) and 0.1 ml bacteria. Tubes were included in which the uterine washing was replaced by 1% horse serum in HBSS and in which the neutrophils were replaced by 0.2 ml HBSS (B). The tubes were rolled at 37° C for 2 h when triplicate serially diluted (10 x) drops (20 ul) were placed on blood agar plates. After incubation overnight at 37° C, the percent survival of bacteria was calculated by:

A/B x 100%.

Haemolytic complement activity

Pig red blood cells (PRBC) suspended in barbitone buffered saline (pH 8.6) were sensitised with a subagglutinating titre of rabbit anti-PRBC serum and incubated for 1 h at 37° C. The pH of washings was adjusted to 8.6 by addition of 1 M NaOH. Washings (0.5 ml) were added to 1.1 ml barbitone buffered saline and 150 ul sensitised PRBC. One hundred percent lysis was calibrated from a tube containing 0.04% ammonia solution and 150 ul sensitised PRBC. Negative controls were included in which barbitone buffered saline was substituted for either washings or the rabbit anti -PRBC serum. In addition, tubes were included which contained heat-treated (56° C for 30 min) washings or serum. Positive controls were provided by addition of serially diluted fresh horse serum. The tubes were incubated at 37° C for 30 min and then spun at 2000 x g for 5 min at 4° C. The degree of haemolysis was measured in the supernatant using a spectrophotometer at 541 nm. Results were calculated as a percentage of the tube containing ammonia solution.

Plasma progesterone and oestradiol-17β assays

These radioimmunoassays were performed using the validated procedures described by Watson et al (1986c).

Statistical analyses

Results were compared between horses by a Student's t test. Blood and uterine neutrophil function within horses were compared with a paired t test and correlations were analysed using Pearson's correlation test. Means are expressed $\pm$ SEM. Results were considered significant when $p < 0.05$.

RESULTS

Elevated plasma progesterone (> 1 ng/ml) concentrations were measured in 8 of the resistant and 7 of the susceptible mares. Elevated plasma oestradiol (> 10 pg/ml) with basal concentrations of progesterone were measured in 6 resistant mares and 7 susceptible mares and 3 of the susceptible mares were in winter anoestrus.

Blood neutrophil migration

Blood neutrophils from susceptible mares were significantly worse ($p < 0.01$) at migrating towards a chemoattractant than neutrophils from resistant mares (Fig. 1). Neutrophils from susceptible mares were also significantly worse ($p < 0.05$) at random migration (Fig. 1). There was no correlation between random and directional migration within mares ($r = 0.3$). Three mares from each group had elevated concentrations (> 1 ng/ml) of plasma progesterone, 3 from each group were in winter anoestrus and the remainder had elevated concentrations of plasma oestradiol (> 10 pg/ml) with basal concentrations of plasma progesterone.

Chemotactic activity of serum

Neutrophils migrated significantly ($p < 0.01$) further towards serum from susceptible mares (1.25 ± 0.03 mm) than towards serum from resistant mares (1.09 ± 0.05 mm).

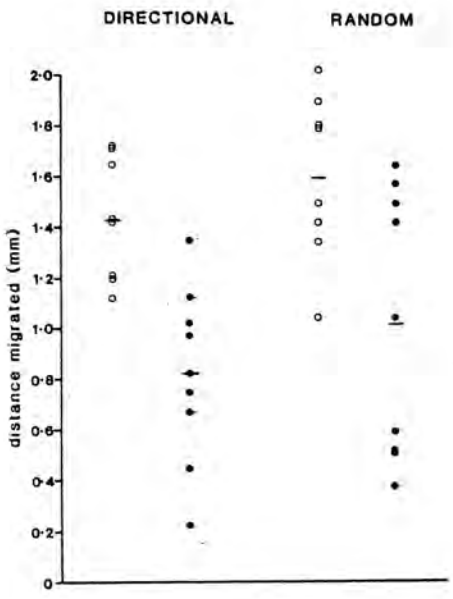

Fig. 1. Directional and random migration under agarose of blood neutrophils from resistant (open circles) and susceptible (closed circles) mares

Phagocytosis by blood and uterine neutrophils

Following induction of uterine infection, within 18 h the 4 mares classified as resistant had eliminated the infection. However, the washings from the 4 susceptible mares contained high numbers of S. zooepidemicus. One of the susceptible mares yielded inadequate numbers of live neutrophils from uterine washings to be included in the phagocytosis assay. Overall, there was no significant difference in the abilities of blood neutrophils from resistant and susceptible mares to phagocytose yeast blastopores (Table I). In contrast, uterine neutrophils from susceptible mares were markedly worse at

phagocytosis than those from resistant mares ($p < 0.01$). In resistant mares, uterine neutrophils were significantly better at phagocytosis than blood neutrophils ($p < 0.01$) but in susceptible mares uterine neutrophils were significantly worse than blood neutrophils ($p < 0.05$).

Pre-incubation of blood neutrophils with bacteria significantly ($p < 0.05$) reduced the neutrophils' ability to phagocytose yeast blastospores (1.8 ± 0.13 blastospores/neutrophil) compared with neutrophils from the same horse which had not been in contact with bacteria (2.4 ± 0.20 blastospores/neutrophil).

TABLE I

Phagocytosis of yeast blastospores by neutrophils from the blood and uterus of mares susceptible and resistant to persistent endometritis

% neutrophils ingesting $\geq$ 2 yeast blastospores					
Susceptible mares	blood neutrophils	uterine neutrophils	Resistant mares	blood neutrophils	uterine neutrophils
A	55	17	E	38	56
B	37	21	F	28	52
C	54	8	G	35	70
D	16	ND	H	19	49

ND = not determined

Yeast opsonisation by uterine washings

In the opsonisation assay, substituting PBS for washing resulted in very low numbers of yeast blastospores (0.2 ± 0.05/cell) being ingested; thus addition of washings enhanced phagocytosis. Washings from susceptible mares were significantly better at opsonising yeast blastospores ($p < 0.05$) than washings from resistant mares (Fig. 2). At the time of sampling 4 of the 17 susceptible mares were infected with beta-haemolytic Streptococci.

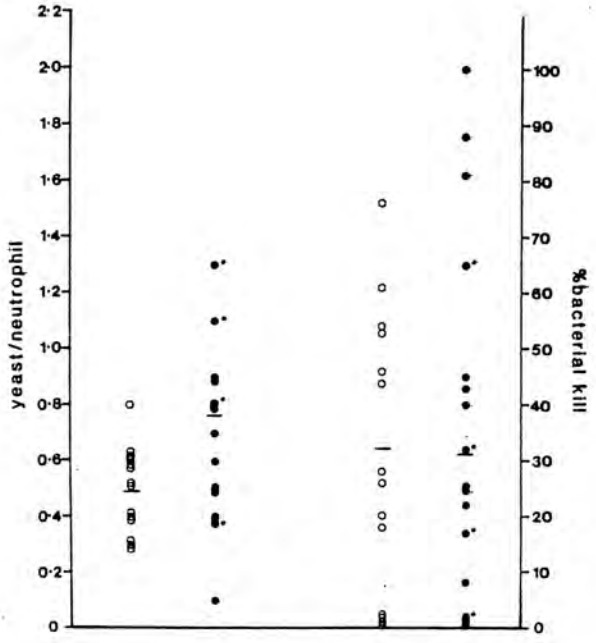

Fig. 2. Promotion of phagocytic and bactericidal activity of neutrophils by uterine washings from resistant (open circles) and susceptible (closed circles) mares.
* These washings were infected with pathogenic bacteria

Bacterial opsonisation by uterine washings

There was a wide range in the ability of washings to promote bactericidal activity of neutrophils and there was no significant difference between susceptible and resistant mares (Fig. 2). Washings from 4 infected mares had

similar activity to those from uninfected mares. There was no correlation between opsonisation of yeast and killing of bacteria (r = 0.198).

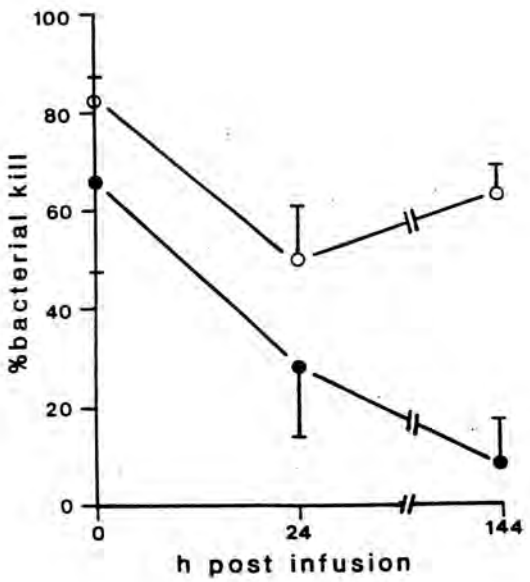

Fig. 3. Promotion of bactericidal activity of neutrophils by uterine washings from 4 resistant (open circles) and 3 susceptible (closed circles) mares after intrauterine infusion of glycogen
Each point represents the mean. Vertical bars represent SEM

Induction of acute endometritis

Infusion of glycogen as a non-immunological irritant resulted in a significant reduction in bactericidal activity of neutrophils suspended in washings collected from resistant mares between 0 and 24 h ($p < 0.05$) and from

susceptible mares between 0, 24 and 144 h ($p < 0.05$), (Fig. 3). Although there was no difference initially in bactericidal activity of neutrophils suspended in washings from the two groups, by 144 h, washings from susceptible mares were significantly worse at promoting bactericidal activity than washings from resistant mares ($p < 0.01$). All 3 susceptible mares succumbed to intra-uterine infection (2 with E. coli and 1 with beta-haemolytic Streptococci) by 144 h whereas the resistant mares remained uninfected.

Haemolytic complement activity

Haemolytic complement activity was detected in uterine washings. Concentrations were very low in washings from the 14 resistant mares (0.1 ± 0.07% haemolysis) whereas in susceptible mares a significantly ($p < 0.001$) greater degree of haemolytic activity was measured (24.4 ± 7.91%). The mean haemolytic activity in susceptible mares was equivalent to 18% of the activity present in serum and the maximum activity measured in washings was 40% of that of serum. Induction of acute endometritis by infusion of glycogen in 3 susceptible (A, B, C,) and 4 resistant (E, F, G, H) mares caused a very slight elevation in 2 of the resistant mares by 24 h after infusion. In the susceptible mares high degrees of haemolysis were measured between 0 and 144 h in 2 of the 3 mares (Table II). Heat treatment of washings and serum reduced haemolysis by 96 to 100% . When barbitone buffered saline was substituted for washings or for the rabbit anti-PRBC serum there was no haemolysis.

TABLE II

Haemolytic complement activity in uterine washings after intrauterine infusion of glycogen

	% haemolysis						
	Susceptible Mares			Resistant Mares			
hours after infusion	A	B	C	E	F	G	H
0	2	86	2	0	0	0	1
24	85	1	0	0	0.4	0.5	0
144	2	73	0	0	0	0	0

DISCUSSION

We have shown that, compared with resistant mares, blood and uterine neutrophils from mares susceptible to endometritis have lower migratory and phagocytic capacities respectively. Uterine secretions from susceptible mares were more effective than secretions from resistant mares at opsonising yeast blastospores, but not bacteria. After induction of acute inflammation, however, secretions from susceptible mares were markedly worse at promoting bactericidal activity of neutrophils than secretions from resistant mares, but, in the secretions from susceptible mares, there was no evidence of any deficiency of haemolytic complement activity.

The ability of neutrophils to respond to chemotactic stimuli and to migrate through tissue is very important in reaching the site of infection. Defects in blood neutrophil migration have been reported in human patients prone to recurrent infections (Quie and Cates, 1978; Chastel et al, 1980). Similarly impaired migration of blood neutrophils has been demonstrated in rats with a focus of inflammation and was attributed to 'counter irritation' (Normann et al., 1985). In another study, however, no difference was found in locomotion of blood neutrophils from susceptible and resistant mares towards a chemoattractant, but migration of uterine neutrophils from susceptible mares was significantly reduced (Liu et al, 1985). In this latter study migration was measured through micropore filters which assessed deformability of neutrophils rather than migration as observed under agarose (Wilkinson, 1982) and could account for the apparent discrepancy in results.

The increased chemotactic activity of serum from susceptible mares contrasts with results from infection-prone humans where decreased chemotactic activity was the commonest defect of serum (Hakansson and Venge, 1980). The enhanced chemotactic activity of serum may reflect the increased chemotactic activity which is present in uterine secretions from endometritic mares (Blue et al, 1984).

In the susceptible mares, uterine neutrophils demonstrated a low capacity for phagocytosis. In these mares, uterine infection persisted from infusion of bacteria until collection of uterine washings 18 h later, whereas by this time infection had been completely eliminated from resistant mares. We have demonstrated that bactericidal activity reduced the capacity of blood neutrophils to phagocytose yeast blastospores. It is possible, therefore, that the phagocytic capacity of uterine neutrophils collected from susceptible mares was compromised due to persisting uterine infection rather than acting as a primary causal factor.

After induction of uterine inflammation, washings from susceptible mares may have been deficient in specific antibody, non-specific bactericidal

proteins or complement. As the susceptible mares had succumbed to bacterial endometritis by 144 h, specific and non-specific opsonins may have been removed with bacteria after centrifugation of the uterine washings. Alternatively, toxic products of oxidative metabolism produced by bactericidal activity of neutrophils (Rosen and Klebanoff, 1979) may have been present in washings from susceptible mares acting to decrease neutrophil function in vitro.

Opsonisation of yeast blastospores by washings from susceptible mares was greater than for washings from resistant mares. This assay depends on deposition of C3b on the yeast cell walls by the alternate pathway of complement activation before efficient phagocytosis of yeast blastospores can take place and has been used clinically to test the alternate pathway in serum samples (Soothill and Harvey, 1976). It seems, therefore, that generation and deposition of C3b were not deficient in secretions from susceptible mares. In contrast, for cytolytic effects on bacteria and for lysis of erythrocytes all components of the complement pathway are required (Lepow, 1971). After induction of acute endometritis there was only a small increase in haemolytic activity in washings from resistant mares - the largest increases occurring between 1 and 6 h after infusion in resistant mares (Watson, unpublished data). Higher activity was present in washings from susceptible mares and was probably related to the degree of inflammation with its associated leakage of serum into the uterine lumen. However, these results differ from those of Asbury et al. (1984), who used sheep red blood cells and reported the absence of haemolytic complement activity from uterine washings after induction of acute endometritis. Other workers, however, have questioned the use of sheep erythrocytes to demonstrate the lytic activity of equine complement (Barta et al, 1973); this may explain the apparent lack of haemolysis in the assay used by Asbury and co-workers (1984).

The present study demonstrated reduced migration by blood neutrophils and possible reduction in phagocytosis by uterine neutrophils from susceptible mares. It also showed a possible deficiency in promotion of neutrophil bactericidal activity by uterine secretions from susceptible mares which was not due to a deficiency in the potential for generation of opsonising complement components.

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Measurement by ELISA of equine alpha-1-proteinase inhibitor in uterine flushings from mares

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An enzyme linked immunosorbent assay (ELISA) was developed and used to estimate the concentrations of the serine proteinase inhibitor, α_1 proteinase inhibitor (API), in uterine flushings recovered from mares at different stages of the oestrous cycle and before and after the induction of experimental endometritis. There was a significant increase in the concentrations of API and albumin relative to total protein in flushings recovered during oestrus compared with dioestrus but no difference was observed in the concentrations of these proteins relative to total protein before and after the induction of endometritis. A regression analysis revealed a significant correlation between the concentrations of albumin and API in the flushings examined, suggesting that the API was derived entirely from serum and was not produced locally in the uterus.

ALPHA-1-PROTEINASE inhibitor (API) is a member of the serine proteinase inhibitor (serpin) group of plasma proteins which regulate the inflammatory cascades, including coagulation, complement activation and the kallikrein pathway. The serpins are also important directly in protecting the tissues of the body from degradation by proteolytic enzymes (Carrell 1986). In the horse four isoforms of API are recognised (Patterson et al 1991) compared with only one in man (also known as α_1-antitrypsin). The Spi-1 isoform of equine API is the closest functional equivalent of human α_1-antitrypsin and exhibits the greatest anti-elastase activity (Patterson et al 1991, Potempa et al 1991).

A deficiency of API is recognised as a cause of several disease syndromes in man, including premature emphysema and liver disease. It is thought that the tissue damage which results in the development of premature emphysema occurs as a result of an imbalance between API and its target enzyme neutrophil elastase. This imbalance results in an excess of protease with respect to its inhibitor, in the alveolar lining fluid, which is then capable of degrading connective tissue components such as elastin and collagen. A functional excess of elastase, leading to tissue destruction, may also occur in people who are not API-deficient in circumstances in which there is a local overwhelming release of proteases or API is inactivated by oxidising agents such as reactive oxygen species released from activated neutrophils (Janoff 1985).

In the horse no gross deficiency of API has been reported but inflammatory conditions such as endometritis and chronic obstructive pulmonary disease (COPD) are characterised by large influxes of neutrophils into the affected organ (Beech 1975, Kenney 1978, Kaup et al 1990). Under these conditions neutrophil degranulation leading to the release of neutrophil elastase may result in a local excess of neutrophil elastase relative to API. It is hypothesised that a prolonged or recurrent exposure to excess levels of neutrophil elastase may contribute to the permanent damage to the endometrium, including periglandular fibrosis, which is recognised as a cause of reduced fertility in older mares that have experienced repeated episodes of endometritis (Kenney 1978).

The liver is known to be the major source of plasma API in man and this is probably also the case in the horse, because API has been localised in equine hepatocytes by immunohistochemistry (Winder et al 1989). API is a small glycoprotein, with a molecular weight of 51,000 in man and 55,000 for equine Spi-1, similar in size to albumin. As a result it can readily diffuse through the interstitial fluid and into the fluid lining of tissue cavities. However, it is also hypothesised that API is produced locally in tissues by other cell lines. Evidence to support the possibility of local production includes the demonstration of the synthesis and secretion of API by human macrophages and monocytes in vitro (Perlmutter et al 1985) and the localisation of API in macrophages in the lungs of both normal and diseased horses (Winder et al 1989).

The objectives of this study were first to develop an ELISA to detect API in equine body fluids, secondly to investigate the potential role of proteases and inhibitors in equine disease states, and thirdly to determine the levels of API in uterine flushings recovered from cycling mares and mares with experimentally induced endometritis. It was also hoped to determine whether API was synthesised by the equine uterus.

Materials and methods

Purification of API and preparation of a polyclonal antibody

The Spi-1 isoform of API was purified, with some residual esterase contamination, from equine serum (API phenotype LL) by the method of Potempa et al (1991). The mixture was separated by sodium dodecyl sulphate polyacrylamide gel electrophoresis (SDS-PAGE) and the Spi-1 band was excised and electro-eluted.

Antiserum to the Spi-1 API was raised in a sheep by inoculating the animal three times over a period of six months with 50 μg Spi-1 API, the first injection being in complete Freund's adjuvant and the subsequent injections in incomplete Freund's adjuvant. The immune serum obtained was first dialysed against 10 mM phosphate buffer and then applied to a DE52 anion exchange column. The IgG_1 fraction eluted with 30 mM sodium chloride. Sheep $F(ab)_2$ anti – Spi-1 API was prepared by digesting the IgG_1 fraction with pepsin (50:1 immunoglobulin:pepsin) for 18 hours at 37°C. Gel filtration with a Sephadex G100 column was used to separate the $F(ab)_2$ fragments from the remaining IgG and Fc fragments and the $F(ab)_2$ fraction was further purified on a DE52 anion exchange column. Finally sheep $F(ab)_2$ anti-horse Spi-1 API IgG_1 antibodies were affinity purified on a Sepharose 4B column containing the Spi-1 protein complexed to rat mast cell protease II (Pemberton et al 1993) as a ligand. The purified antibody was coupled to horseradish peroxidase as described by Wilson and Nakane (1978). The solutions of antibody were stored at –40°C until required.

To purify API from serum in batches an affinity column was prepared with the purified antibody linked to CNBr-activated Sepharose 4B (1 g). Equine serum (API phenotype LL) was diluted 10-fold with phosphate buffered saline (PBS), then passed down the column. The retained protein was eluted in 0·1M citric acid, 0·5M sodium chloride and the pH was restored to neutrality by the immediate addition of 1M Tris. The four isoforms of API accounted for more than 90 per cent of the retained protein eluted from the affinity column as determined by SDS-PAGE.

The pooled API fractions were concentrated by ultrafiltration, buffer exchanged on Sephadex G25 to 5 mM phosphate, pH 6·5 (containing 0·01 per cent sodium azide) then chromatographed on a Mono-Q ion exchange column in the same buffer. The Spi 1 inhibitor eluted as a sharp peak at 100 mM sodium chloride with minimal contamination when visualised by SDS-PAGE.

ELISA for equine API

Affinity purified sheep anti-API (1 μg ml^{-1}) in 0·1M carbonate/bicarbonate buffer was used to coat the wells (50 μl per well) of micro-ELISA plates (M129B Dynatech). After incubation at 4°C overnight the plates were washed six times with PBS containing 0·05 per cent Tween 20 (PBS-T20). Samples and standards were diluted with PBS-T20 containing 4 per cent bovine serum albumin (BSA, Sigma Chemical) and 50 μl of each standard or sample were added per well of the plate. The affinity purified Spi-1 isoform of API (phenotype LL) was used as a standard for all assays over the range 0·5 to 8 ng ml^{-1}. After the addition of the samples and standards the plate was incubated at room temperature for one hour and then washed six times. Fifty microlitres of a 1/250 dilution of sheep $F(ab)_2$ anti-horse API IgG conjugated with horseradish peroxidase was added per well and the

FIG 1: a) Coomassie blue-stained Native PAGE gel showing in lane 1 albumin depleted equine serum (UU phenotype) and in lane 2 the α-1-proteinase inhibitor isoforms eluted from the Spi-1 affinity column (note two subtypes of Spi 3)

FIG 1: b) Native PAGE gel incubated with trypsin, then treated with N-acetyl phenylalanine-β-naphthyl ester and Fast Blue Salt B. Trypsin inhibition by API isoforms is shown by clear zones in the gel, samples as for Figure 1a (note that only three of the isoforms inhibit trypsin)

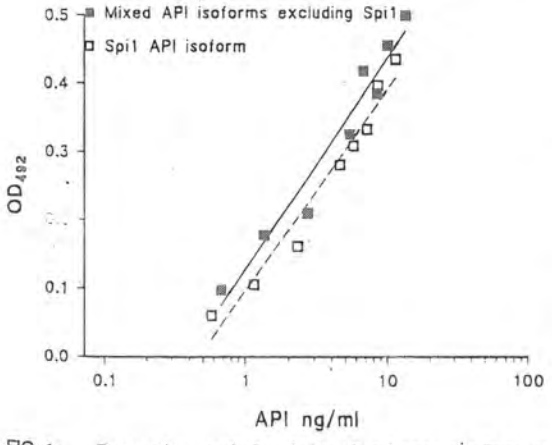

FIG 1: Regression analysis of the absorbance readings at 492 nm for different concentrations of Spi-1 and mixed isoforms of α-1-proteinase inhibitor (excluding Spi-1) measured by ELISA

plate was incubated for a further hour at room temperature followed by a final six washings in PBS-T20. The substrate o-phenylenediamine (Sigma Chemical) in the presence of hydrogen peroxide was used to initiate colour development in the wells, the reaction being terminated by the addition of 25 µl per well of 2·5M sulphuric acid. The colour intensity was measured at 492 nm (Titertek Multiscan Reader, Flow Laboratories).

Specificity of the polyclonal antibody

Polyclonal antibodies raised against Spi-1 formed a single line of precipitation on double diffusion against horse serum. However the same antibodies, affinity purified against Spi-1/RMCPII complex and covalently linked to Sepharose 4B, bound all equine API isoforms as determined by Native PAGE (Fig 1a) and by inhibitor gel analysis (Fig 1b) (Pemberton and John 1993). When Spi-1 and a separate mixture of the other API isoforms were prepared by anion exchange chromatography on Mono-Q and subjected to the proposed ELISA the results showed that the polyclonal antibody recognised both the purified Spi-1 isoform of equine API and a mixture of the other API isoforms equally (Fig 1c). An analysis of the two regression

lines shown in Fig 1c suggests that they were not significantly different from one another.

When samples containing Spi-1 and elastase complexes were applied to the anti Spi-1 affinity column, SDS-PAGE of the eluted proteins showed that the polyclonal antibody recognised complexed API in addition to the native API isoforms. This suggests that in common with monoclonal antibodies raised against human native API the polyclonal antibody did not distinguish between native, inactivated or complexed API (Herion et al 1984).

Measurement of total protein and albumin

Estimates of protein concentration were made using the bicinchoninic acid protein assay (Pierce and Warriner, Chester) and the bovine serum albumin (fraction V) supplied with the kit was used as a standard.

A radial immunodiffusion method was used to estimate albumin concentrations (Mancini et al 1965). Rabbit anti-horse albumin antiserum (Nordic Laboratories) was used at dilutions of 1/100 or 1/200 in 1 per cent agar in PBS. Horse serum albumin (essentially globulin free, Sigma Chemical) was used as a standard. The assays were incubated for 24 hours at room temperature.

Uterine flushings

Paired uterine flushings were taken from five mares during oestrus and mid-dioestrus and also from previously ovariectomised mares. Flushings were also obtained from six mares immediately before the induction of experimental endometritis with *Streptococcus zooepidemicus* and two days after treatment of the induced infection with either sterile water or 2 g of ampicillin in sterile water (McDonnell and Watson 1993). The flushing technique was based on that described by Zavy et al (1978) and is briefly described below. The mares were prepared for the collection of uterine flushings by cleaning the perineal area three times with a povidone-iodine scrub after the rectum had been emptied of faeces. A sterile 24 Ch foley catheter with a 30 ml cuff (Warne Franklin) was passed through the cervix and 50 ml of sterile PBS was flushed through the catheter into the uterus and allowed to reflux under gravity into a sterile collection cup.

The volume of fluid recovered was measured and a small sample was streaked on to a horse blood agar plate. The remaining fluid was centrifuged at 200 *g* for 20 minutes. The supernatant was divided into 1 ml aliquots and frozen at –60°C until analysis. The cell pellet was resuspended in PBS and a cytospin preparation was stained with Diff Quik (Baxter Healthcare).

Statistical analysis

The API and albumin concentrations are expressed as a proportion of total protein. The relative API and albumin concentrations taken from the same mares during oestrus and dioestrus or before and after infection were compared by a paired *t* test. A Student's *t* test was used to compare the relative API, albumin concentrations and volumes of fluid recovered for the samples collected from the ovariectomised mares and those in oestrus or dioestrus. A regression analysis was used to examine the correlation between the concentrations of API and albumin in all the samples.

Results

Oestrus and dioestrus uterine flushings

The mean (SEM) percentage recovery of uterine fluid tended to be lower and more varied for the ovariectomised mares and those in oestrus (67·6 [11·8] and 61·0 [8·4] per cent) than for the mares in dioestrus (82·0 [4·3] per cent) but the differences were not statistically significant. The variation observed in the recoveries at oestrus was probably due to a tendency for the fluid to reflux past the cuff of the catheter when the cervix was widely dilated, as has previously been observed by Strzemienski and Kenney (1984). No bacteria were cultured from any of the cycling mares and the percentage of neutrophils in the cytospin preparations ranged from 0 to 15 per cent.

The ELISA technique measured API in the recovered uterine flushings successfully. There was a significant increase in the mean (SEM) concentrations of API ($P=0·037$) and albumin ($P=0·026$) relative to total protein in the oestrus flushings compared with the dioestrus flushings from the same mare (for albumin the difference was 31 [9] and for API it was 3·9 [1·3] $\mu g\ mg^{-1}$ total protein) (Table 1) although the variation in total protein concentration was larger between individual mares during oestrus. There were no significant differences between the mean (SEM) albumin concentrations of flushings recovered from the ovariec-

TABLE 1: Relative concentrations of albumin and API in uterine flushings (μg mg^{-1} total protein) from mares in different reproductive states

Mare	Stage of cycle	Albumin	API
1	Oestrus	61	1·8
2	Oestrus	84	9·0
3	Oestrus	88	3·7
4	Oestrus	67	3·6
5	Oestrus	49	6·5
	Mean (± SEM)	69 (7)	4·9 (1·3)
	Dioestrus	61	0·7
	Dioestrus	31	1·1
	Dioestrus	46	1·4
4	Dioestrus	33	1·1
5	Dioestrus	24	0·4
	Mean (SEM)	39 (7)	0·9 (0·2)
A	Ovariectomised	57	2·9
B	Ovariectomised	71	5·7
C	Ovariectomised	73	3·9
D	Ovariectomised	148	7·5
	Mean (SEM)	87 (21)	5·0 (1·0)

TABLE 2: Relative concentrations of albumin and API in uterine fluids from mares before the induction of endometritis and after treatment with either water or ampicillin. Protein concentrations are μg mg^{-1} total protein

Mare	Treatment	Before infection Albumin	API	After infection Albumin	API
M	Water	245	36·5	218	20·8
CA	Water	178	3·3	95	0·7
CH	Water	152	3·3	387	14·5
R	Water	100	5·4	113	3·7
S	Water	138	16·2	101	8·2
E	Water	378	5·0	575	16·1
M	Ampicillin	158	3·3	262	7·0
CA	Ampicillin	31	0·6	192	9·2
CH	Ampicillin	102	0·9	186	1·2
R	Ampicillin	78	2·1	40	0·3
S	Ampicillin	148	8·1	54	1·0
E	Ampicillin	216	5·2	317	4·6

tomised, oestrus or dioestrus mares (87 [21], 69 [7] and 39 [7] μg mg^{-1}, respectively) or between the mean API concentration in flushings from oestrus or ovariectomised mares (4·9 [1·3] and 5 [1] μg mg^{-1} total protein, respectively). However, there was significantly more API in the flushings from the ovariectomised animals than from those in dioestrus (5 [1] and 0·9 [0·2] μg mg^{-1} total protein, P=0·029).

The results from all the mares in oestrus, dioestrus or after ovariectomy were pooled and a regression analysis was used to compare the concentrations of API and albumin in each flushing. The analysis revealed a significant positive correlation between the concentrations of API and albumin (r=0·79, P=0·0007) (Fig 2).

Flushings taken from mares before the induction of experimental endometritis and after treatment with water or ampicillin

The percentage of neutrophils in cytospin preparations of uterine flushings increased from less than 15 per cent of uterine cells before the induction of endometritis to 60 to 100 per cent after treatment with either ampicillin or water, indicating that an inflammatory response had been invoked in all the mares (McDonnell and Watson 1993). There were no significant differences between the concentrations of either API or albumin (when corrected for total protein) before

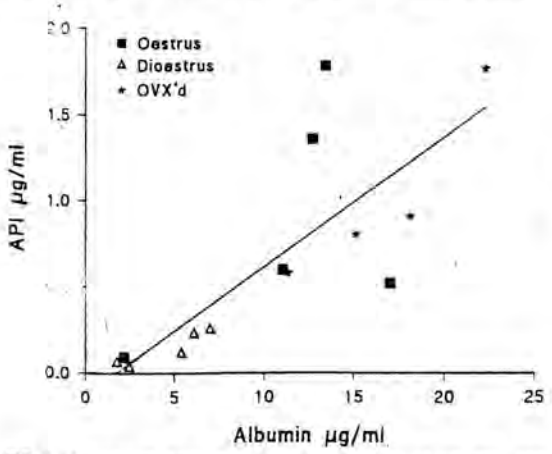

FIG 2: Correlation between the concentrations of albumin and alpha-1-proteinase inhibitor measured in uterine flushings from ovariectomised (OVX'd) mares and mares in oestrus and dioestrus (r=0·79)

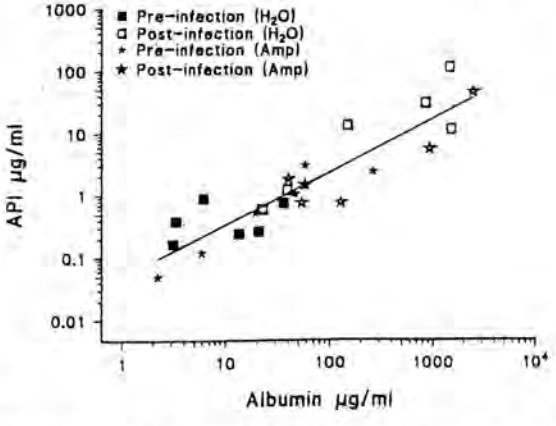

FIG 3: Correlation between the concentrations of albumin and alpha-1-proteinase inhibitor in uterine flushings from mares before the induction of endometritis and after treatment with water or ampicillin (r=0·91)

infection or after treatment of the same mares with either water or ampicillin (Table 2). However, there was wide variation in both albumin and API concentrations between the individual mares.

As had been found for the cycling mares there was a significant positive correlation between the concentrations of API and albumin in each flushing (Fig 3) ($r=0{\cdot}91$, $P<0{\cdot}0001$).

Discussion

The existence of a relatively common deficiency state in man has stimulated the measurement of API concentrations by a variety of techniques, including ELISA, radial immunodiffusion and the measurement of trypsin inhibitory activity, in a range of human transudates including alveolar lining fluid, uterine and follicular fluid, gingival crevicular fluid and synovial fluid. However, although API and API-$\alpha_2\beta_1$-glycoprotein complexes have been detected immunohistochemically in equine tissues (Winder et al 1989, 1990) there have been few quantitative investigations of the potential importance of API-protease imbalance in disease states in the horse or other species. The present study aimed to establish a sensitive ELISA which could be used to measure API concentrations in equine body fluids. The polyclonal antibody raised against the equine Spi-1 isoform of API made it possible to estimate total API levels in samples of equine uterine flushings.

The results indicated that there was more API and albumin relative to total protein flushings taken from mares during oestrus than during dioestrus. This result differs from the results of a previous study in the mare which observed no change in either the total or relative albumin concentrations in uterine flushings during the oestrous cycle (Widders et al 1985). The results of the present study must be interpreted cautiously because the method of uterine flushing did not allow the degree of dilution of the uterine fluid to be assessed. The apparent reduction in the concentration of API and albumin observed in dioestrus may therefore be due either to an increased protein synthesis by the endometrium (leading to an increase in the total non-serum protein) or to a reduced leakage of serum proteins, or to changes in the concentrations of serum proteins. Although some studies have observed no changes in protein concentrations in the reproductive tract fluids during the cycle (Blue et al 1982, 1984, Strzemienski and Kenney 1984) other studies have reported that total protein levels in the oviduct (Engle et al 1984) and uterus of the mare increase in dioestrus (Zavy et al 1978, Widders et al 1985, McDowell et al 1987) in comparison with oestrus. The increase in protein concentration in the uterine fluids observed in dioestrus can be partially explained by the synthesis of progesterone-dependent proteins by the endometrium (Zavy et al 1982, McDowell et al 1982, Hansen et al 1985). There is also experimental evidence to suggest that in the mare the volume of oviductal secretions is greater in oestrus than in dioestrus (Engle et al 1970) and this suggests that the mare is similar to females of other domestic species, for example, cattle and sheep, in secreting less fluid into the reproductive tract during dioestrus. Other tissues show similar changes in protein leakage in response to changes in plasma hormone concentrations. For example, a study of the levels of serum proteins in bovine milk observed that the concentrations of API and albumin increased when the animals were injected with oestrogen, an increase which was attributed to an increase in the rate of leakage of serum protein into the mammary gland (Saad et al 1990).

Serum albumin levels do not appear to vary with the stage of the cycle in mares (Widders et al 1985) and studies of the effect of hormonal status on serum API levels in women (measured by using radial immunodiffusion or trypsin inhibitory activity) have demonstrated no significant variation with the oestrus cycle (Lieberman et al 1971, Kueppers et al 1972). However, when synthetic oestrogens, but not progestagens, were administered serum API levels increased (Laurell et al 1967, Lieberman et al 1971). The influence of serum API on the levels of API in uterine fluid has not been fully explained and examinations of fluids from human reproductive tracts have suggested that API levels decrease in cervical mucus at around the time of ovulation (Schumacher and Pearl 1968) and that proteinase inhibitor concentrations (Casslen and Ohlsson 1981) and trypsin inhibitory activity increase in uterine fluid in the luteal phase (Fazleabas et al 1987). Proteinase inhibitors may have a different physiological function in the human uterus compared with their function in the equine uterus. It has been suggested that in women inhibitors could have a role in controlling the timing of the invasion of the endometrium by the blastocyst (a process which

involves tissue proteolysis) (Fazleabas et al 1987). In contrast, the equine blastocyst does not implant into the endometrium and there may therefore be no comparable functional need for inhibitors in equine uterine fluids.

In this study more than one flushing was obtained from each mare during an oestrous cycle. Widders et al (1985) suggested that when animals were repeatedly flushed during an oestrous cycle the protein concentrations measured in a sample were affected by the day on which the sample was collected. However, in this study, in which samples were collected only twice from any mare, the same trend in API and albumin levels relative to total protein was observed independent of whether the first sample was taken during oestrus (mare 1, 2, 5) or dioestrus (mare 3, 4).

In the mares in which endometritis was induced there was an increase in the albumin and API concentrations (Fig 3) and therefore in the total protein concentration in the uterine flushings collected after the introduction of bacteria. An increase in the total protein concentration in the uterine fluid of mares with endometritis has previously been reported (Strzemienski and Kenney 1984, Williamson et al 1983, 1987, Pycock and Allen 1990) and attributed to increased vascular permeability, the synthesis of proteins by the endometrium, and the products of neutrophils and cell breakdown. In this study, the observation that the concentrations of albumin and API with respect to total protein were not changed suggests that the increase in the protein concentration in uterine fluid observed two days after the treatment of the induced endometritis began can be attributed primarily to an increase in vascular permeability as a result of the inflammatory process.

The other aim of this study was to establish whether there was a local production of API. Various serpin like proteins have been identified as being synthesised by the uterus in sheep, cattle and rats (Percival and Starcher 1988, Ing and Roberts 1989, Leslie and Hansen 1991) but the only studies which have looked for API in uterine fluids (in women) have suggested that the API present is derived entirely from serum (Casslen 1986, Fazleabas et al 1987). In the present study, the concentrations of serum protein albumin and API were highly correlated in the samples taken at different stages of the cycle and after infection (Figs 2 and 3), providing strong evidence that there was no local production of API.

This study has made it possible to define some reference data for total API concentrations in the equine uterus and it has shown that API is not produced locally by the endometrium. Further studies will be required to investigate the potential role of protease/inhibitor balance in endometritis and to investigate whether serum concentrations of total and functional API and the different isoforms are affected by the stage of the oestrous cycle of the mare.

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Review Article

Uterine defence mechanisms in mares resistant and susceptible to persistent endometritis: A review

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IT is widely acknowledged that mares bred under intensive systems of management achieve disappointingly low levels of fertility. Foaling rates vary from 50 to 80 per cent (Day 1939; Dewes 1973; Laing and Leech 1975; Sullivan *et al* 1975; Rossdale and Ricketts 1980), with an almost linear drop in fertility with increasing age (Jeffcott *et al* 1982).

An important cause of sub-fertility, especially in older, multiparous mares is the condition of persistent endometritis. Some degree of chronic inflammatory changes within the endometrium, in the form of progressive glandular degenerative change and stromal fibrosis, seems to be an inevitable consequence of multiparity (Kenney 1978; Ricketts 1978). However, some mares apparently develop a local immunological deficiency and fail to resolve the acute endometritis stimulated at coitus by the introduction of opportunistic bacterial pathogens and environmental contaminants directly into the uterus (Hughes and Loy 1975). Other sources of uterine contamination include parturition and reproductive examination, even when performed as hygienically as possible. These mares remain persistently infected and are termed 'susceptible' mares. In contrast, genitally-normal, or 'resistant' mares eliminate infection within 72 h of intra-uterine infusion of bacteria (Peterson, McFeely and David 1969).

Certain conformational characteristics of mares tend to predispose to persistent endometritis. Defective shape and angulation of the vulva are more common in larger breeds and predispose to pneumovagina (Allen and Newcombe 1979). Air within the reproductive tract is an important cause of inflammation and infection (Roberts 1986) and, in some cases, appears to be associated with eosinophils in uterine histology and cytology specimens (Slusher, Freeman and Roszel 1984).

Clinical diagnosis relies on palpation of the genital tract *per rectum*, vaginoscopic examination, detection of neutrophils in endometrial smears and isolation of a heavy growth of pathogenic bacteria from a uterine swab or washing (Rasbech 1965; Wingfield Digby and Ricketts 1982; Couto and Hughes 1984; Asbury 1987).

By far the most commonly isolated micro-organisms from mares with endometritis are ß-haemolytic streptococci (Elliot, Callaghan and Smith 1971; Conboy 1978; Shin, Lein, Aronson and Nusbaum 1979; Wingfield Digby and Ricketts 1982). Other isolates recovered from a significant number of cases include *Escherichia coli*, *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Corynebacteria* species. Confirmation is obtained by microscopical examination of an endometrial biopsy (Kenney 1978; Ricketts 1978). Acute endometritis is characterised by intense neutrophilic infiltration of the endometrium sometimes in association with degenerative changes of the lumenal epithelium. In cases of persistent endometritis, there is evidence of chronic mononuclear cell infiltration, glandular degenerative changes and stromal fibrosis and it often presents with a superimposed acute endometritis.

Degrees of endometritis present in biopsies have been graded from I to III (Kenney 1978). Mares with Category III endometria have significantly lower foaling rates (11 per cent) than Category I mares (68 per cent). Even the light to moderate leucocytic infiltration with chronic glandular degeneration seen in Category II mares is associated with a significant reduction in foaling rates (51 per cent). Reduction in conception rates in animals with endometritis is probably caused by the presence of neutrophils in the uterine lumen. Neutrophils secrete substances which are cytotoxic to gametes and embryos and consequently prevent implantation (Vickery and Bennett 1968; Waites and Bell 1982). Additionally, post coital endometritis which persists beyond the fifth day after ovulation may initiate luteolysis (Neely *et al* 1979), resulting in loss of luteal support for the maintenance of pregnancy.

The uterus responds to infection by a rapid influx of neutrophils and serum proteins, including immunoglobins, into the uterine lumen (Peterson *et al* 1969; Williamson, Penhale, Munyua and Murray 1984; Watson *et al* 1987c). Mechanical responses of smooth muscle and mucociliary clearance also assist in clearing bacteria (Evans *et al* 1986). During the acute phase of inflammation, in addition to the chemotactic substances released

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by bacteria themselves (Ward 1968), immunoreactive leukotriene B_4 (LTB_4) a potent chemotaxin (Lees, Dawson and Sedgewick 1986; Watson, Stokes and Bourne 1987a) is present in elevated concentrations in uterine secretions and may assist in recruiting neutrophils from the bloodstream. Uterine secretions collected from infected mares have been shown directly to contain specific chemotaxins which stimulate neutrophil migration *in vitro* (Blue, Blue, Kenney and Merritt 1984) and also chemokinetic substances, such as prostaglandins, which stimulate random neutrophil migration (Lees *et al* 1986; Watson *et al* 1987a). Once neutrophils have reached the infected uterine lumen, however, they lose the ability to migrate *in vitro* under agarose (Watson *et al* 1988) probably due to auto-oxidative injury associated with bactericidal activity (Smith and Lumsden 1983) and to deactivation by exposure to high concentrations of chemotaxins (Gallin, Wright and Schiffmann 1978). In contrast, the uterine neutrophils retain their ability to migrate through millipore filters and it has been reported that neutrophils from susceptible mares are significantly less able to migrate than neutrophils from resistant mares (Liu *et al* 1985) apparently due to a loss of deformability. Recent work has shown a premature migration dysfunction in uterine neutrophils from susceptible mares (Liu *et al* 1986) at 12 h after infection. At other inflammatory sites, however, it is thought that the ability to migrate towards a chemotactic substance is not important once neutrophils have reached the site of inflammation (Wilkinson 1982).

There is no evidence that the infiltration of neutrophils into the uterine lumen is delayed in susceptible mares after intra-uterine infusion of bacteria (Asbury *et al* 1982; Watson 1986). However, *in vitro* studies on blood neutrophils from susceptible mares have revealed a reduction in both chemotactic and chemokinetic locomotion under agarose compared with blood neutrophils from resistant mares (Watson, Stokes and Bourne 1987b). This observation could be secondary to the presence of a focus of chronic inflammation as has been reported in rats (Normann, Schardt and Sorkin 1985). By contrast, in another study, no differences in migration of blood neutrophils were found between resistant and susceptible mares when the millipore filter technique was used (Liu *et al* 1985).

Once neutrophils have reached the uterine lumen, their capacity to ingest and kill invading micro-organisms is critical in the rapid elimination of infection. When uterine neutrophils were collected 12 to 18 h after infusion of bacteria, the phagocytic ability *in vitro* of uterine neutrophils from susceptible mares was significantly less than uterine neutrophils from resistant mares (Cheung, Liu, Walsh and Miller 1985; Watson *et al* 1987b). However, these results appeared to be dependent on the method of collection of uterine neutrophils, because when sterile culture filtrate was used, no defects were found in the phagocytic capacity of uterine neutrophils from susceptible mares: in fact, phagocytosis by these neutrophils appeared to be enhanced (Asbury and Hansen 1987). Bactericidal activity reduces the phagocytic capacity of neutrophils (Watson *et al* 1987b). Therefore, any factor which favoured persistence of bacteria in the uteri of susceptible mares rather than in resistant mares could have affected *in vitro* phagocytosis in the former two studies.

The process of opsonisation is of central importance in phagocytosis of micro-organisms. Opsonisation of particles changes the ingestion by phagocytes from a slow or negligible basal rate to a rapid one. Antibody attached to the surfaces of bacteria can provide binding sites for phagocytes to attack the offending organisms. It is probable that, in the absence of complement, only antibodies of the IgG class are opsonically active (Menzel, Jungfer and Gemsa 1978). In cervicovaginal secretions from cattle, IgG acts as an opsonin, whereas both IgG and IgA immobilise bacteria (Corbeil *et al* 1974).

The presence of complement significantly increases opsonisation and bactericidal activity of neutrophils (Glovsky, Alenty and Ghekiere 1978; Menzel *et al* 1978) and enables IgM to act as an opsonin. Although IgM is more efficient than IgG at opsonisation, the relative inability of IgM to gain access to tissue inflammatory sites usually renders IgG the more important opsonin. Although immunoglobins G, A and M have been detected in uterine secretions from mares (Kenney and Khaleel 1975; Asbury, Halliwell, Foster and Longino 1980; Mitchell *et al* 1982; Widders, Stokes, David and Bourne 1984), IgM is usually either undetectable or present only at very low concentrations. Evidence for local uterine synthesis of immunoglobins has been provided by measurement of a higher ratio of immunoglobins to total immunoglobin or albumin in secretions than in serum (Mitchell *et al* 1982; Widders *et al* 1984), immunohistological identification of plasma cells (Mitchell *et al* 1982; Widders, Stokes, David and Bourne 1985a) and demonstration of *in vitro* synthesis of antibody by endometrial explants (Watson 1987). The presence of a well developed local secretory immune system has been confirmed by local immunisation studies which showed a significant uterine, but minimal systemic, response (Widders *et al* 1985b). It is probable, however, that in the inflamed endometrium the contribution from serum is considerably increased as a result of increased serum leakage into the uterine lumen. Mares with persistent endometritis have higher concentrations of immunoglobins in uterine secretions than genitally-normal mares (Asbury *et al* 1980; Williamson, Dunning, O'Conner and Penhale 1983; Widders *et al* 1984). When levels of specific antibody to *Streptococcus zooepidemicus* were measured in uterine secretions from persistently-infected mares, they were found to be similar to those from resistant mares which had been infected experimentally with *S zooepidemicus* (Watson 1986). Despite apparently normal levels of antibodies, both chemiluminescent (Asbury *et al* 1982) and bactericidal studies (Watson *et al* 1987b) have found that secretions from susceptible mares are less effective at opsonising bacteria than secretions from resistant mares. Furthermore, heat-treated culture supernatant, containing antibodies synthesised by endometrial explants from susceptible mares, was less effective as an opsonin than culture supernatant of endometrial tissue from resistant mares (Watson 1986). In contrast, workers have reported increased opsonisation of bacteria by secretions collected from the uteri of susceptible mares (Hansen and Asbury 1987). It is likely that these conflicting results are caused by the different techniques employed to measure neutrophil activity. It is also possible that substances may be present in biopsy supernatant or uterine secretions which directly stimulate or suppress neutrophils and therefore interfere with interpretation of opsonisation results. Even the pH of the secretions may alter neutrophil function. Furthermore, other substances, such as lactoferrin, may act as non-specific opsonins to increase the adherence of phagocytes to bacteria and chemical bactericidal substances may kill bacteria directly (Strzemienski, Do and Kenney 1984).

The rate of opsonisation is increased dramatically by the presence of complement proteins. One study has failed to detect the presence of functional complement in uterine secretions and only found evidence of complement in its degraded form (Asbury, Gorman and Foster 1984). However, using a different assay system, there is recent evidence that there is more haemolytic complement in secretions from endometritic than from genitally-normal mares (Watson *et al* 1987b). In addition, the presence of C3 in uterine secretions has been demonstrated indirectly by the ability of uterine secretions to opsonise yeast blastospores *in vitro* (Watson *et al* 1987b). Similarly, Brown, Hansen and Asbury (1985) reported that complement was present in secretions collected 6 h after intra-uterine inoculation of *S zooepidemicus*.

The cells involved in antigen presentation within the mare's uterus have not yet been identified. In other species however, macrophage-like or dendritic cells appear to possess the Ia antigen which is associated with the ability to present antigen

(Head and Gaede 1986; Tabibzadeh, Bettica and Gerber 1986). Differences in patterns of Ia antigen expression have been identified by immunological staining techniques in the endometria of women with normal uteri and those with chronic endometritis (Tabibzadeh *et al* 1986). In mares, the only data available which compare antigen uptake in mares with persistent endometritis and genitally-normal mares have been obtained using intravenous injection of ^{125}I-labelled polyvinyl-pyrrolidone. No differences in rate of removal from the blood were detected in the two groups of mares (Watson and Stokes 1987). However, as local antigen uptake by endometrial cells was not studied, the presence of a localised defect in antigen uptake cannot be ruled out.

Various treatment regimes have been adopted in attempts to cure endometritis in combination with surgical correction of any anatomical defects. Most commonly, either intra-uterine lavage with saline (Shideler, Voss and Squires 1984; Threlfall and Carleton 1986), systemic or local antibiotic treatment or infusion of antiseptic solutions (Threlfall 1976; Woolcock 1980) have been employed. However, these therapies have a limited success rate and can be associated with yeast superinfection (Neely 1985). Another approach has been to boost the natural defence mechanisms of the uterus by systemic treatment with oestrogen (Azzie 1975) or intra-uterine inoculation of culture filtrate of *S zooepidemicus* (Couto and Hughes 1985). Plasma or colostrum have been infused into the uterus of affected mares to provide a rich source of exogenous immunoglobins and complement (Asbury 1984; de Gannes 1985) and uterine curettage has also been employed to induce regeneration of superficial endometrial layers (Ricketts 1985). Enzyme preparations such as pepsin and papain have been used locally to treat pyometra as has intra-uterine dimethylsulphoxide (Threlfall and Carleton 1986). In horses where artificial insemination is acceptable, another method of dealing with these mares is to try to prevent infection at breeding by utilising antibiotic treated semen extender or by the minimum contamination technique (Kenney, Bergman, Cooper and Morse 1975). The success of these treatment regimes for endometritis has proved very difficult to interpret because of lack of adequate control mares and absence of a suitable experimental model on which to perform controlled experiments. Recently workers in Colorado have used ovariectomised mares treated with progesterone as models for susceptible mares (Colbern *et al* 1987a). Using this model they have shown that intra-uterine infusion of plasma did not significantly alter the course of introduced infection (Colbern *et al* 1987b).

Although recent work has elucidated some of the immune defence mechanisms within the uteri of mares, there is much scope for future research in the areas of antigen presentation and processing within the uterus, especially in view of the different responses elicited by sperm, bacteria and the developing embryo. Circulating and local hormonal concentrations have a major influence on uterine defence and should be studied in relation to development of susceptibility in mares with persistent endometritis.

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An immunohistological study of MHC Class II expression and T lymphocytes in the endometrium of the mare

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Summary

The distribution of T lymphocytes and of cells bearing MHC Class II antigens in the endometrium of the mare was studied using an avidin–biotin–peroxidase staining method. The cells within the endometrium which expressed MHC Class II were macrophages, lymphocytes, monocytes, dendritic cells, epithelial cells and endothelial cells. MHC Class II expression increased significantly ($P<0.05$) in the luminal epithelium and tended ($P=0.0573$) to increase in the subepithelial layers during oestrus. Numbers of T lymphocytes did not differ between oestrus and dioestrus. MHC Class II expression and T lymphocyte numbers were not significantly different in samples collected on Day 14 of oestrus and on Day 14 of pregnancy. The presence of endometritis greatly increased MHC Class II expression and T lymphocyte numbers within the endometrium.

Introduction

Persistent uterine infection is a major cause of subfertility in the mare. Some mares appear to be incapable of eliminating the microorganisms which gain entrance to the uterus at parturition, at coitus, during veterinary gynaecological examinations, or due to poor perineal conformation of the mare and these mares remain persistently infected. Mares with persistent endometritis have greater numbers of neutrophils and mononuclear cells within the uterine lumen (Watson *et al.* 1987) and endometrium (Kenney 1978) than do mares with normal uterine immune defence mechanisms. Even mares with normal uterine immune defence mechanisms will succumb to establishment of intrauterine infection if the microorganisms are introduced when the endometrium is under the influence of progesterone (Ganjam *et al.* 1982).

The normal endometrium of the mare, in common with other mucosal surfaces, is capable of mounting a local immune response to invading pathogens (Widders *et al.* 1986; Watson 1988), thereby maintaining a sterile environment within the uterus. Although routine histological examination of the endometrium of the mare has revealed the presence of lymphocytes, plasma cells and macrophages, all of which are essential in most immune responses, there have been no detailed studies of immune cell populations within the endometrium of the non-pregnant mare. To initiate most immune responses, it is necessary to have cells present with the capacity for antigen recognition, processing, and presentation to immunoregulatory T lymphocytes. These cells may be recognised immunohistologically by their expression of surface major histocompatability complex (MHC) Class II antigens. Variable expression of MHC Class II antigens has been observed in the endometrial epithelium of women during the menstrual cycle (Tabibzadeh *et al.* 1986a). Because of the increased susceptibility of the uterus of the mare to infection during dioestrus, immunohistological techniques were used to ascertain whether MHC Class II expression and T lymphocyte numbers within the endometrium vary during the oestrous cycle. Biopsy specimens were also collected from mares with endometritis and from mares in early pregnancy to see whether these two conditions were associated with changes in MHC Class II expression.

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Materials and methods

Animals

Fifteen light horse mares, 3–11 years of age and weighing between 450 and 550 kg, were used to study the effect of ovarian hormones on the normal endometrium. All of these mares had Category I endometria by the criteria of Kenney (1978). An additional 6 horse mares were included which had endometritis. One of these mares was 4 years old and had acute endometritis with neutrophil infiltration into the endometrial stroma that persisted for approximately 3 weeks and then resolved spontaneously. Three mares had chronic persistent bacterial endometritis, with Category III endometria, and had been infertile for several years. The remaining 2 of the 6 mares were 4 and 11 years of age, did not exhibit bacterial growth from swabbings of the endometrium, and had Category II endometria with cystic glandular distension and mononuclear cell infiltration.

Endometrial biopsy specimens

Mares were teased daily with a stallion and the ovaries were examined daily by ultrasonography *per rectum* to monitor follicular development. Endometrial biopsy samples were collected from the 15 mares with normal endometria during 1 or 2 consecutive oestrous cycles. Samples were obtained at 1 of 6 periods: Day 3 of oestrus, day of ovulation (Day 0), and Days 3, 6, 10 and 14 of dioestrus. Samples were collected from some of the mares during

th oestrus and dioestrus (the remaining mares were not available repeated biopsy sample collection) so that differences could be mpared within mares. Individual mares were not sampled over veral cycles in case the biopsy procedure induced changes in igen expression in the endometrium. Biopsy specimens were lected during dioestrus from the mares with endometritis.

Seven of the mares with Category I endometria were ificially inseminated. At Day 14 after ovulation, the presence a conceptus was confirmed by ultrasonographic examination *rectum* and endometrial biopsy samples were collected. The opsy specimens were placed in O.C.T. (Miles Inc., Elkhart, IN, A) and snap-frozen in liquid nitrogen. The tissue was then red at -70°C until further processing.

Three consecutive 5-μm-thick cryostat sections were placed gelatin-coated slides, air dried, and fixed in acetone at 4°C for ecs before storage under desiccated conditions for up to 1 week -70°C. Before being stained, the sections were fixed again in d acetone for 5 mins.

munoperoxidase staining procedures

e 3 consecutive sections were stained by one of 3 methods. The t section was stained with haematoxylin and eosin (H & E). ner sections were stained by an avidin–biotin complex (ABC) ning method (Hsu *et al.* 1981) using antibodies for MHC Class II igens (TH14B; Davis *et al.* 1987) and T lymphocytes (EqT3; att *et al.* 1988). The EqT3 antibody has been shown to be a rker for the T lymphocyte CD5 antigen (Kydd and Antczak 91). Primary monoclonal antibodies were purchased from VRMD . (Pullman, WA, USA) and avidin and biotinylated peroxidase s (Vectastain Kit, Pk 4002) and biotinylated horse anti-mouse munoglobulin G were purchased from Vector Laboratories urlingame, CA, USA). Initially, the sections were incubated with normal horse serum for 20 mins at room temperature. tibodies were used at the following concentrations: anti-MHC II, 5 μg/ml and anti-EQT3, 15 μg/ml, and were incubated on the tions for 30 mins at 37°C. The second antibody was applied for mins at room temperature. Endogenous peroxidase activity was moved by immersing the slides in a bath of methanol and 0.01 M- sphate buffered saline (PBS, pH 7.2; 1:1 v:v) containing 0.3% drogen peroxide for 20 mins. Next, the avidin–biotin complex gent was added and left on the slide for 60 mins at room perature. Di-amino benzidine tetrahydrochloride (0.06%) 0.03% hydrogen peroxide in 0.05 M-TRIS [Tris(hydroxy-thyl)aminomethane] buffer, pH 7.6, was used as the final strate and resulted in a brown reaction product after 5–7 mins. tions were counterstained with haematoxylin, dehydrated and unted in Histoclad (Clay Adams, Parsippany, NJ, USA).

ntrols

ntrol sections were included in which the first or second ibody was replaced by PBS, horse serum, or monoclonal ibody to oxytocin (VCB-Bioproducts, Braine-l'Alleud, gium). Any endogenous peroxidase activity was detected by ubating the fixed sections with the substrate solution. Any logenous avidin-binding activity was detected by incubating ed sections with the avidin–biotin complex for 1 h and then posing it to the substrate solution after washing.

antitation

e proportions of positive cells in 5 high-power fields (x40) in regions of the endometrium, the stratum compactum perficial layer of the endometrium) and the stratum ngiosum (deeper layer of the endometrium), were calculated tive to the total number of cells per field. Aggregations of phocytes (>10 cells) were not included in the analysis because as impossible to count individual cells within the aggregates.

Due to the patchy nature of the epithelial staining, the extent of staining was graded qualitatively as follows: Grade 0, absence of staining; Grade 1, single cells stained within the epithelium; Grade 2, diffuse patches of epithelial staining.

Statistical analysis

Data used for statistical analysis derived from biopsy samples collected from 7 mares during oestrus (Day 3 of oestrus or on the day of ovulation) and dioestrus (Days 6 or 10). Analysis of variance for repeated measures was used to evaluate differences between stage of cycle and endometrial layers for each of the two antigens. The variables examined were the average number of cells per field, the average number of stained cells per field and the total proportion of stained cells. The percentage of stained cells was evaluated using an arc sine transformation. MHC Class II expression in the luminal epithelium during oestrus and dioestrus was analysed by a paired t test. A significance level of 0.05 was used for all statistical tests.

Results

Identification of MHC Class II cells through the oestrous cycle

MHC Class II staining was identified in macrophages, monocytes, dendritic cells, lymphocytes, blood vessel endothelium and the uterine luminal epithelium. MHC Class II antigen was absent from the glandular epithelium except in pathological states. In the luminal epithelium the presence of staining was frequently associated with the presence of subepithelial aggregations of lymphoid cells.

Macrophages were present throughout the endometrium and within the lymphoid aggregates. By comparison with the consecutive H&E sections, some macrophages did not express MHC Class II antigen or were only slightly stained. Monocytes were seen very occasionally and always exhibited paler staining than did macrophages. Class II-positive dendritic cells were found irregularly in the endometrium.

MHC Class II-positive lymphocytes were distributed throughout the endometrium, though with greater density in the stratum compactum than in the stratum spongiosum. Aggregates of the MHC Class II-positive lymphocytes also occurred, comprising the majority of the cells in the lymphoid aggregates with fewer macrophages and very few dendritic cells. Occasional MHC Class II-positive lymphocytes were seen within the epithelium. Such intraepithelial lymphocytes were uncommon, and were not included in the cell counting.

Identification of T lymphocytes through the oestrous cycle

The positively stained T lymphocytes were found mostly in the stratum compactum and irregularly in the stratum spongiosum. Cells appeared to be of two morphological types. The resting T lymphocyte had a small, circular, intensely-stained nucleus with scant cytoplasm and frequently showing the characteristic perinuclear halo of cytoplasmic retraction. The second type had a slightly larger, paler nucleus with more abundant cytoplasm. Most of the cells in lymphoid aggregates that were positive for MHC Class II antigen were T lymphocytes.

Effect of cycle stage and pregnancy

Figures 1, 2 and 3 show expression of MHC Class II antigen in the endometrium and luminal epithelium and T lymphocyte numbers in the endometrium at various stages of the cycle and during early pregnancy. Samples collected during oestrus or during early dioestrus appeared to have more cells which expressed MHC Class II antigen than samples collected during mid-dioestrus (Fig 1). By Day 14 (late dioestrus), expression had again increased. Statistical analysis was performed on data from 7 of the mares which had been

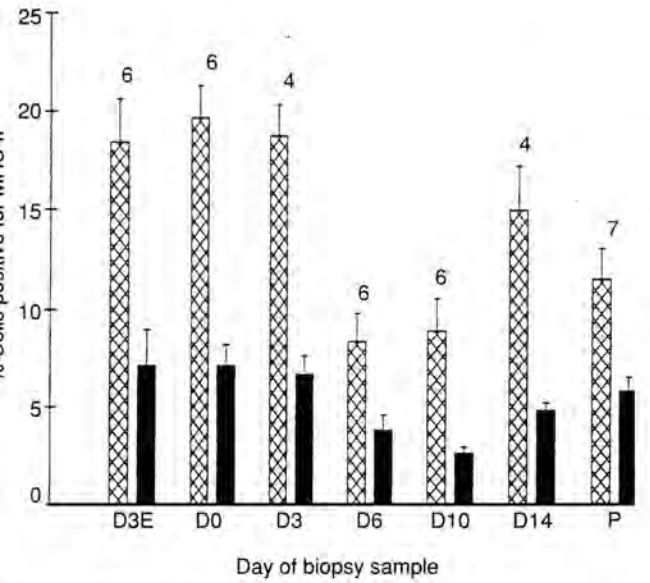

Fig 1: MHC Class II expression (mean ± sem cells positive for MHC Class II) in the stratum compactum (cross-hatched bars) and stratum spongiosum (closed bars) of the endometrium of the mare at stages of the oestrous cycle and at Day 14 of pregnancy. D3E = Day 3 of oestrus; D0 = day of ovulation; D3–14 = Days of dioestrus; P = Day 14 of pregnancy. Figures above bars represent number of mares.

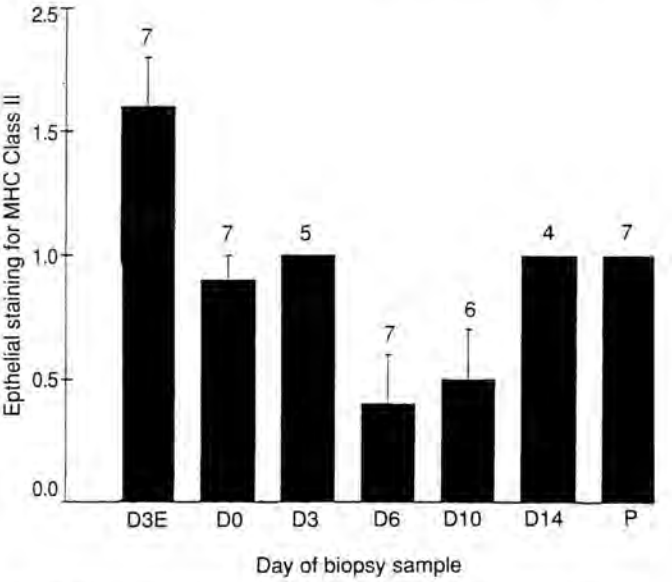

Fig 2: Degree of epithelial expression of MHC Class II antigen (mean± sem) at stages of the oestrous cycle and at Day 14 of pregnancy in the endometrium of the mare. 0 = no staining; 1 = single cells stained; 2 = diffuse patches of staining; D3E = Day 3 of oestrus; D0 = day of ovulation; D3–14 = Day of dioestrus; P = Day 14 of pregnancy. Figures above bars represent number of mares.

sampled during both oestrus and dioestrus. For both antigens the number of stained cells and the proportion of stained cells was significantly ($P<0.05$) greater in the stratum compactum than in the stratum spongiosum. There were no significant differences between cyclic and pregnant mares in total numbers and relative proportions of stained cells. However, during oestrus a higher proportion of cells expressed MHC II antigen ($P=0.0573$) than during dioestrus. Samples from mares when they were in oestrus had significantly ($P<0.05$) more intense staining of the luminal epithelium for MHC Class II antigen (1.2 ± 0.16) than samples from the mares when they were in dioestrus (0.3 ± 0.21). There were no significant differences in MHC Class II expression by luminal epithelial cells between mares sampled at Day 14 of pregnancy and Day 14 of dioestrus (Fig 2).

Immunohistological studies on 6 mares with endometritis

All 3 mares with persistent endometritis (Category III) had intense, diffuse MHC Class II-positive staining of the luminal epithelial cells. These 3 mares either had very diffuse MHC Class II-positive cell infiltration in the stratum compactum or had many large lymphoid aggregates which were almost entirely MHC Class II-positive, consisting mainly of T lymphocytes. Two of the 3 mares exhibited patches of MHC Class II staining in glandular epithelium.

The 2 mares with chronic degenerative changes within the endometrium (Category II) had cystic gland distension and periglandular fibrosis. These mares showed a high level of MHC Class II expression in cells around the distended glands and patchy MHC Class II expression in the luminal epithelium. Only a few of the MHC Class II-positive cells around the distended glands were T lymphocytes. Most of the MHC Class II cells were blood vessel endothelial cells and some macrophages were also present.

Compared with the endometria of the normal (Category I) mares, the endometrium of the mare with acute endometritis showed an increase in MHC Class II expression. This mare had diffuse patches of heavily stained luminal epithelium.

Intraepithelial lymphocytes were far more common in the luminal epithelium from the mares with endometritis than from mares with normal (Category I) endometria. Lymphoid follicles

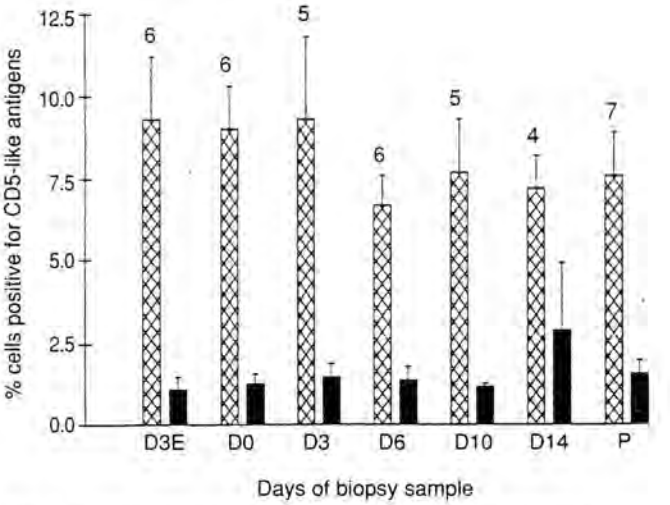

Fig 3: T lymphocytes (mean ± sem) in the stratum compactum (cross-hatched bars) and stratum spongiosum (closed bars) of the endometrium of the mare at stages of the oestrous cycle and at Day 14 of pregnancy. D3E = Day 3 of oestrus; D0 = day of ovulation; D3–14 = Day of dioestrus; P = Day 14 of pregnancy. Figures above bars represent number of mares.

were not detected in the endometria of any of the mares.

There was no specific staining in the control sections for either of the antigens. Endogenous peroxidase activity and endogenous avidin binding activity were absent from the sections.

Discussion

We have described the distribution of MHC Class II expression within the endometrium of the non-pregnant mare and have shown a tendency for increased expression during oestrus in the subepithelial layers and a significant increase in the luminal

epithelium. As far as we can ascertain the present study is the first which has attempted to quantitate cells expressing MHC Class II and lymphocytic infiltration of the endometrium. The endometrium becomes oedematous during oestrus (Kenney 1978), and significant trends may be masked by this natural phenomenon if only numbers of positive cells in high-power fields are counted. Accordingly, in our study, positive cells were expressed as percentages of total cells. By contrast, MHC Class II expression on the epithelium was assessed qualitatively as the density of this tissue is not affected by cycle stage. The luminal epithelium of endometrium from non-pregnant women has also been reported to express MHC Class II antigen (Morris *et al.* 1985; Tabibzadeh *et al.* 1986a, b). This antigen was absent from the glands in the superficial layers of the endometrium, but was present in glands of the basalis layer of the human endometrium. By contrast, MHC Class II expression was always absent from glands in the endometrium of mares with Category I endometria, but patchy staining was present in some of the glands of 2 of the mares with infected uteri. It is known that immunological stimuli induce expression of MHC Class II antigen (Barclay and Mason 1982). In women, the superficial layers of the endometrium are lost monthly during menses exposing deeper gland epithelium. By contrast, in the mare the duct and gland epithelium are not the first regions to contact antigen entering the uterus, which probably explains the absence of MHC Class II expression on these two regions. The presence of MHC Class II staining in the glands of 2 of the mares with endometritis may indicate the deep-seated nature of the infection.

In the mare, as in women (Tabibzadeh *et al.* 1986a) and rats (Head and Gaede 1986; Zheng *et al.* 1989), MHC Class II expression was increased during periods of high circulating concentrations of oestrogen, ie at oestrus. The endometrium is a highly hormone-responsive organ and it is conceivable that MHC Class II expression may be hormonally regulated. The pattern of MHC Class II expression within the mare endometrium resembles the pattern of concentrations of oestrogen receptors (Tomanelli *et al.* 1991), further suggesting a link between MHC Class II expression and hormonal regulation. Expression of MHC Class II antigen by epithelial cells has been associated with areas of enhanced immune response (Barclay and Mason 1982; Selby *et al.* 1983) and therefore may be related to the great resistance of the uterus to infection during oestrus.

Inducibility of MHC Class II antigens is an important feature of many epithelia. Inflammatory stimuli are associated with induction of MHC Class II expression in epithelial cells both *in vitro* (Cerf-Bensussen *et al.* 1984) and *in vivo* (Barclay and Mason 1982). Increased expression of MHC Class II has been reported in the endometrial epithelium of women with chronic endometritis (Tabibzadeh *et al.* 1986a). It is known that lymphoid cells are capable of inducing MHC Class II molecules in other cell types (Cerf-Bensussen *et al.* 1984) and one of the products of activated T cells, γ-interferon, induced MHC Class II expression in human endometrial cells *in vitro* (Tabibzadeh *et al.* 1986b). In mares with Category III endometria, the diffuse infiltration of the stratum compactum with lymphoid cells, many of which were T cells, was associated with strong expression of MHC Class II in the overlying epithelium even though the mares were in dioestrus. These MHC Class II-positive epithelial cells may be involved in presentation of antigen to T cells as has been demonstrated *in vitro* using gut epithelial cells (Bland and Warren 1986).

The proportion of T lymphocytes in the mare endometrium did not appear to vary with the stage of the oestrous cycle. Because the proportion of MHC Class II-positive cells tended to increase during oestrus, and because resting equine T lymphocytes express MHC Class II (Crepaldi *et al.* 1986), it would seem that the additional cells were macrophages, monocytes, endothelial cells, and B cells. T lymphocyte numbers greatly increased in mares with persistent endometritis. The lymphoid aggregates in both normal mares and mares with endometritis were composed mainly of MHC Class II-positive cells and most of the lymphocytes appeared to be T lymphocytes. It is notable that the position of the lymphoid aggregates differed between species. In women the aggregates were confined to the deeper regions (Tabibzadeh *et al.* 1986a), whereas aggregates were found throughout the endometrium of the mare. This finding is probably again related to the monthly shedding of the superficial layers of the endometrium in women.

In the present study, there was no significant difference in the MHC Class II expression between Day 14 of pregnancy and Day 14 of dioestrus. Expression of MHC Class II antigens was markedly lower in samples from mares in early pregnancy than in samples from mares with endometritis. Accordingly, it is possible that the endometrium from the pregnant mares was responding not to antigenic stimulation by the developing conceptus but rather to hormonal stimulation from the ovarian follicular development which tends to occur at this time (Sirois *et al.* 1989).

In conclusion, we have shown that MHC Class II expression tended to increase in the endometria of mares during oestrus, which is the time of maximum exposure to antigens and which is known to be the period of maximum uterine resistance. MHC Class II expression was increased in mares with endometritis, presumably in response to antigen exposure.

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Lymphocyte subsets in the endometrium of genitally normal mares and mares susceptible to endometritis

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Keywords: horse; uterus; lymphocytes

Summary

The density and distribution of MHC Class II positive cells nd subpopulations of lymphocytes were studied in the ndometrium of genitally normal mares and mares susceptible o endometritis. In genitally normal mares, more MHC Class I positive cells were present in the epithelium and *stratum ompactum* during oestrus than dioestrus. Significantly more D4+ and CD8+ lymphocytes were present in the *stratum ompactum* than in the *stratum spongiosum*. CD4+ ymphocytes were present in greater numbers than CD8+ ymphocytes in the *stratum compactum* but approximately qual numbers were present in the *stratum spongiosum* and in ymphoid aggregates. Occasional CD4+ and CD8+ cells were een in the luminal and glandular epithelium. Infrequently, B ells were present in the endometrium and were not observed n the epithelium. Numbers of T and B cells did not appear to e affected by cycle stage. In mares with endometritis, the ensities of CD4+, CD8+ and B cells were significantly ncreased. Large aggregates of lymphoid cells which contained pproximately twice as many CD4+ cells as CD8+ cells were resent in the endometrium of these mares and all 3 subclasses f lymphocyte were seen occasionally in luminal and glandular pithelium.

ntroduction

ersistent endometritis is an important cause of subfertility in nares. Persistence of infection within the uterus seems to depend pon both physical clearance of fluid from the uterus after ontamination (Troedsson *et al.* 1993) and on immune defence echanisms (Watson 1988). It is important to establish the istribution and prevalence of lymphocyte subpopulations within ne uterus of the mare in order to understand the immune echanisms responsible for elimination of uterine infection. urthermore, a recent study has shown differences in endometrial eucocytic subpopulations between fertile women and women ith unexplained infertility (Klentzeris *et al.* 1994).

Previous studies have described the distribution of MHC lass II expression and T lymphocytes in the mare endometrium Watson and Dixon 1993; Frayne and Stokes 1994). Since our arlier study, monoclonal antibodies which recognise certain ymphocyte subsets have become available. In the present study, ne density and distribution of cells bearing MHC Class II, CD4+ nd CD8+ antigens and B cells in the endometrium by genitally ormal mares and mares susceptible to endometritis were studied y immunohistology.

Materials and methods

Fifteen genitally normal pony mares, age 3–20 years and weighing 250–450 kg were examined by transrectal ultrasonography daily until ovulation. Endometrial biopsies were collected according to the procedure described by Kenney (1978) between Days 7 and 12 after ovulation (n=7; dioestrus) and between Days 16 and 20 (n=8; oestrus). The biopsies were placed in OCT compound[1] and snap frozen in an isopentane/dry ice slurry. The samples were then kept at -70°C until further processing. On examination of sections stained with haematoxylin and eosin, the endometria were classified as *category I* or *IIA*. Biopsies were also collected during oestrus from 6 mares age 6–18 years with histories of subfertility and recurrent acute bacterial endometritis, and processed as above. Endometria from these mares were classified as *category IIB* or *III*.

Immunostaining procedures

Sections were placed on Biobond-coated[2] slides and air dried for 2 h. The sections were stained by an avidin-biotin complex staining method using monoclonal antibodies for MHC Class II (TH 14B), CD4+ (HB 61A), CD8+ (HT 14A) and B cells (MAC 292). TH 14B, HB 61A and HT 14A were purchased from VMRD (Washington State University, USA) and MAC 292 was kindly donated by Dr Julia Kydd (Animal Health Trust, Newmarket, UK). The specificities of these antibodies have been reported previously (Kydd *et al.* 1994). The avidin-biotin peroxidase kits were purchased from Vector Laboratories[3]. After fixing in acetone for 5 min the slides were washed in phosphate buffered saline (PBS) and incubated with glucose oxidase for 60 min at 37°C to block any endogenous peroxidase staining. After washing again with PBS, the slides were incubated for 15 min with normal horse serum to block nonspecific staining before incubating overnight at 4°C with the primary antibody. The sections were then washed and incubated with the second antibody (horse anti-mouse; horse anti-rat for MAC 292) for 30 min. The avidin-biotin complex was added after washing and the sections were incubated for a further 30 min. Chromagen (3-amino-9-ethylcarbazole:AEC) was added as the final substrate and produced a red reaction product after 10 min. The sections were then washed in tap water, counterstained with Meyer's haematoxylin and mounted for microscopic examination. Control sections were included in which the first antibody was replaced by normal horse serum. No endogenous peroxidase activity was detected in these sections. Sections of lymph node were included on each slide to act as positive controls.

TABLE 1: Immune cell density ($\bar{x}$ ± s.e.m.) in mare endometrium during oestrus and dioestrus and in oestrous mares with recurrent acute endometritis

	Cycle stage					
	Oestrus (n=8)		Dioestrus (n=7)		Endometritis (n=6)	
Antigen	SC	SS	SC	SS	SC	SS
CD4+	3 ± 0.19	1.8 ± 0.25	2.7 ± 0.18	2 ± 0.22	3.8 ± 0.31[a]	3.3 ± 0.2[b]
CD8+	2.4 ± 0.18	1.4 ± 0.18	2.3 ± 0.20	1.9 ± 0.34	3.2 ± 0.17[b]	2.7 ± 0.33[b]
B cell	1.1 ± 0.12	0.8 ± 0.16	1 ± 0.22	0.9 ± 0.14	2.8 ± 0.31[b]	2 ± 0.26[b]

SC = *stratum compactum;* SS = *stratum spongiosum.*
Cell densities were graded on a scale from 0 to 5. Superscripts denote a significant difference from normal oestrous endometrium: [a] P<0.05; [b] P<0.01.

Quantitation

Density of positive staining cells was expressed on a scale of 0 to 5 and graded for the epithelium (for MHC Class II), the *stratum compactum* and the *stratum spongiosum*.

Statistical analysis

Epithelial staining was compared between sections by an unpaired *t* test. The relative cell densities were compared within sections using a paired *t* test. A level of significance of P<0.05 was considered to be significant.

Results

Genitally normal mares

Expression of MHC Class II by the luminal epithelium was greater (P=0.01) in genitally normal oestrous mares ($\bar{x}$ ± s.e.m. = 1.9 ± 0.40) than in dioestrous mares ($\bar{x}$ ± s.e.m.= 1 ± 0.49). Glandular epithelium only rarely expressed MHC Class II. Positive epithelial staining was frequently associated with the presence of subepithelial lymphoid cells (Fig 1). Foci of MHC Class II positive cells were observed occasionally in the *stratum spongiosum* and infrequently in the *stratum compactum* (Fig 2).

Significantly more (P<0.01) CD4+ and CD8+ cells were present in the *stratum compactum* than in the *stratum spongiosum* (Table 1; Figs 3 and 4). CD4+ cells were more numerous than CD8+ cells (P<0.05) in the *stratum compactum* but they were not significantly different in the *stratum spongiosum*. Lymphocytes of both phenotypes were infrequently seen migrating through the luminal epithelium and only rarely seen in the glandular epithelium. Lymphocyte aggregates of approximately equal numbers of CD4+ and CD8+ were present and these were usually larger in the *stratum spongiosum* than in the *stratum compactum*. The aggregates were often next to glands, blood vessels or lymphatics.

Infrequently, B cells were seen in the endometrium of genitally normal mares and no B cells were present in the luminal or glandular epithelium. B cells were not detected in the lymphoid aggregates. Densities of T and B cells did not appear to be affected by cycle stage.

Mares with endometritis

Many MHC Class II+ cells were present in the endometria of mares with endometritis but although there tended to be more, there was no significant difference in expression between infected mares and genitally normal mares.

Mares with endometritis had significantly more CD4+ and CD8+ cells in the endometrium than genitally normal mares. These mares had large lymphoid aggregates within the *stratum compactum* and *stratum spongiosum*. It was noticeable, however, that the aggregates appeared to contain approximately twice as many CD4+ cells than CD8+ cells (Fig 5a and b); CD8+ cells were present more frequently in the luminal and glandular epithelium of mares with endometritis than CD4+ cells.

B cells were scattered throughout the endometrium (Fig 6) and in endometrial lymphoid aggregates and were occasionally seen in luminal and glandular epithelium.

Discussion

In this study, we have documented the distribution and relative frequency of MHC Class II positive cells, CD4+ and CD8+ lymphocytes and B cells in the endometrium of the mare. We have reported in a previous study, the distribution of cells bearing MHC Class II antigens in the equine endometrium at different stages of the oestrous cycle (Watson and Dixon 1993) and these findings were confirmed in the present study.

In the horse, T lymphocyte subsets, CD4+ and CD8+, are mutually exclusive in peripheral blood (Kydd and Antczak 1991; Lunn and Duffus 1991). Cytotoxicity experiments have shown MHC Class I restriction for CD8+ lymphocytes (O'Brien *et al.* 1991) which is similar to other species. In other species, these 2 lymphocyte subsets have different functional roles with CD4+ being helper cells and CD8+ being a cytotoxic/suppressor population (Parnes 1989). The populations of CD4+ and CD8+ cells have not been reported previously in the endometrium of the nonpregnant mare. In mares with a normal endometrium, there were more CD4+ and CD8+ cells in the *stratum compactum* than in the *stratum spongiosum*. Both subsets tended to be present in approximately equal numbers, although in the *stratum compactum* there tended to be more CD4+ than CD8+ cells. By contrast, in human and ovine endometrium, CD8+ cells appeared to be predominant (Kamat and Isaacson 1987; Bulmer *et al.* 1988; Segerson *et al.* 1991). Lymphoid aggregates lacking germinal centres were commonly present in the normal mare endometrium and these consisted of approximately equal numbers of CD4+ and CD8+ cells. In lymphoid aggregates from noninfected human endometrium, CD8+ lymphocytes were usually present in greater numbers than CD4+ lymphocytes (Morris *et al.* 1985; Kamat and Isaacson 1987; Bulmer *et al.* 1988) although in one study equal numbers were found (Tabibzedeh *et al.* 1986).

Only a few scattered B cells were present in the normal mare endometrium and they did not appear to be present in lymphoid aggregates. In other mucosal sites, lymphoid aggregates devoid of germinal centres would normally be expected to consist of B cells. It is probable that the lack of B cells reflects the limited amount of antigenic stimulation received by the endometrium which is

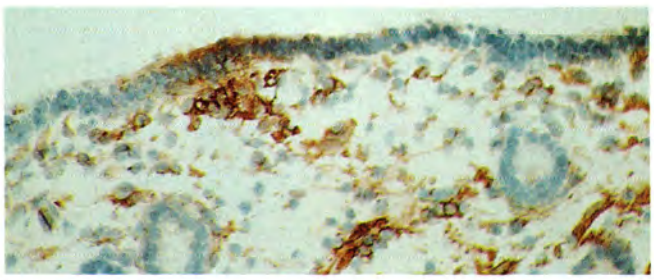

Fig 1: MHC Class II expression in luminal epithelium associated with subepithelial MHC II positive cells in a genitally normal mare (avidin-biotin complex x25).

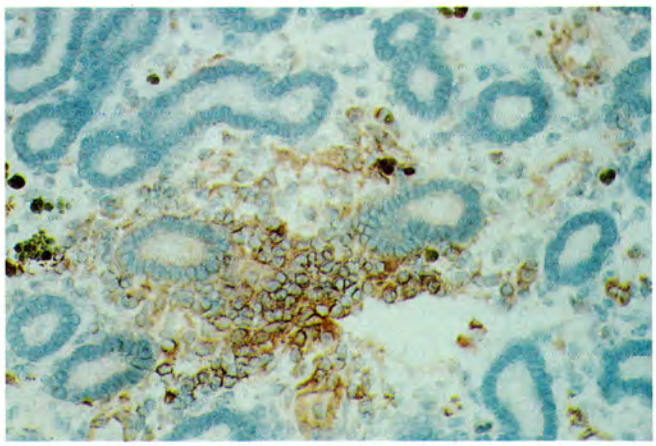

Fig 2: Aggregate of MHC Class II positive cells in the stratum spongiosum *of a genitally normal mare (avidin-biotin complex x25).*

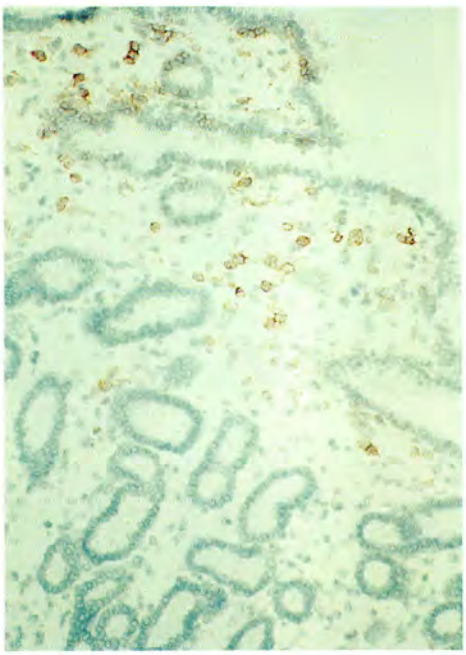

Fig 3: CD4+ cells in the endometrium of a genitally normal mare (avidin-biotin complex x25).

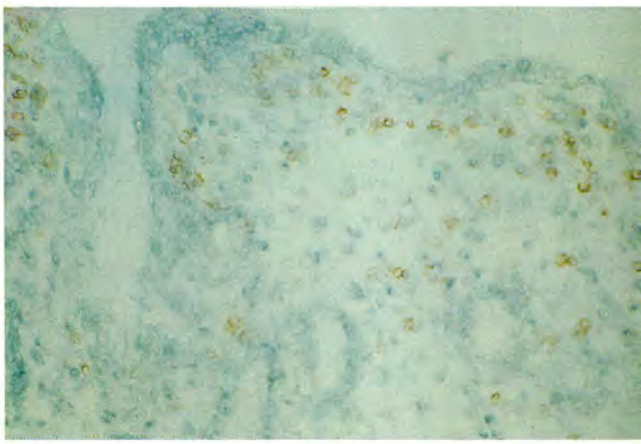

Fig 4: CD8+ cells in the endometrium of a genitally normal mare (avidin-biotin complex x25).

thought normally to be maintained as a sterile environment.

Very few intraepithelial lymphocytes were seen in normal mares and these were either CD4+ or CD8+. No intraepithelial B cells were found in endometrium of normal mares in the present study or in that location in other species (Morris *et al.* 1985; Kamat and Isaacson 1987; Lee *et al.* 1988).

Neither subset of T cell, nor B cells appeared to be affected by stage of cycle in the mare. Because different mares were studied in oestrus and dioestrus it was not possible to perform direct comparisons between cycle stages. However, in another study where paired samples were obtained from the same mares, no differences in CD5+ (pan T) lymphocyte numbers were detected (Watson and Dixon 1993). In the human endometrium, T cells either increased during the secretory phase of the cycle, with CD8+ cells predominating (Bulmer *et al.* 1988), or remained constant (Starkey *et al.* 1991).

In the endometrium of other species, large granular lymphocytes, thought to belong to the natural killer cell lineage, have been identified (Lee *et al.* 1988; King *et al.* 1989; Head *et al.* 1994). These cells have not been documented in the mare endometrium. Accordingly, we stained sections of endometrium from mares with Toluidine Blue which stains granulated cells (Bulmer *et al.* 1991), but although numerous mast cells were identified, no granular lymphocytes were detected. It is possible, however, that these cells are present during pregnancy.

The T cell infiltration in mares with endometritis was so dense, that any subtle differences in infiltration between the *stratum compactum* and *spongiosum* could not be detected. A marked difference was observed in the lymphoid aggregates of infected mares compared with genitally normal mares, in that the aggregates were larger than those of the normal mares and many more CD4+ cells were present than CD8+ cells. It seems probable that mor CD4+ cells, which have a central role in driving and directing th immune response against extracellular bacterial infection b stimulating phagocytosis and B cell antibody production, wer recruited than CD8+ cells. In the immune response, CD8+ cells ar also effector cells but recognise and kill cells expressing foreig antigen and are, therefore, involved more in viral infections.

In the present study, many more B cells were present in th endometria, including lymphoid aggregates, of mares with acut bacterial endometritis. Histological examination of consecutiv sections stained with haematoxylin and eosin suggested that the cell marker may also be recognising a common antigen on som plasma cells. Plasma cell infiltration in mares with endometriti has been reported previously (Watson and Stokes 1988; Waelchl and Winder 1991) and is evidence of immunoglobulin productio In the presence of antigen, B cells can develop into plasma cell with T cell help and, therefore, it is not surprising that there ma

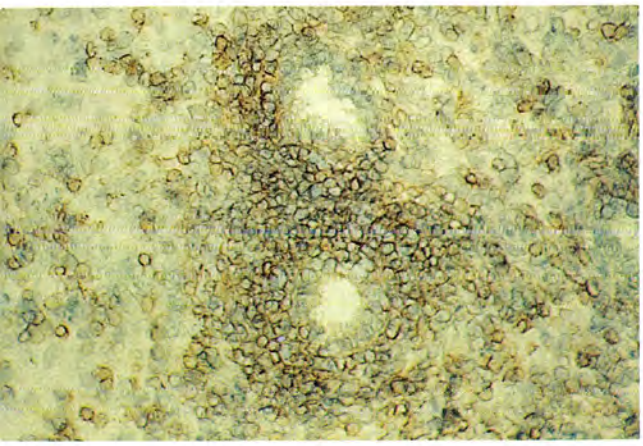

Fig 5a: Aggregate of CD4+ lymphoid cells in a mare with endometritis (avidin-biotin complex x25).

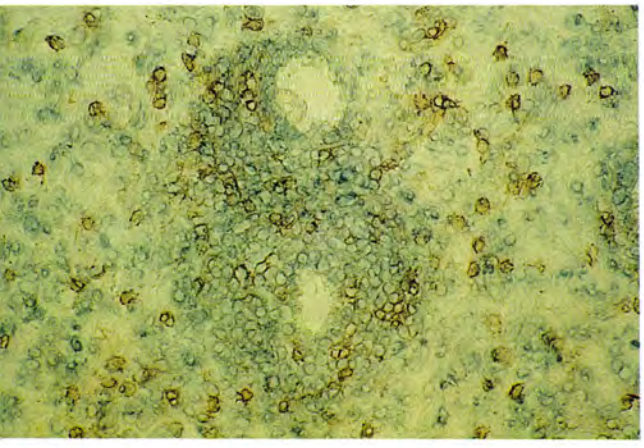

Fig 5b: Consecutive section of aggregate of CD8+ lymphoid cells in a mare with endometritis (avidin-biotin complex x25).

be some antigenic cross-reactivity in the dynamic environment of the immune response. Intraepithelial CD8+ cells and B cells were seen more frequently in the endometrium of mares with endometritis than in genitally normal mares. In the nonpregnant human and bovine endometrium most of the intra-epithelial lymphocytes are CD8+ (Morris *et al.* 1985; Kamat and Isaacson 1987; Cobb and Watson 1995). The role of endometrial intra-epithelial lymphocytes is not known but Morris *et al.* (1985) suggested that, after detecting antigen, these cells could migrate to lymphoid aggregates and to regional and central lymphoid tissue for amplification of the immune response and it has recently been shown that antigen introduced into the pig uterus can activate cells in the local draining lymph nodes (Bischof *et al.* 1994).

In the mare, endometrial tissue contains all the components of mucosal-associated lymphoid tissue seen at other mucosal sites. It is important to recognise the prevalence of lymphocytes and plasma cells in moderate numbers in the endometrium of fertile mares. These cells do not necessarily indicate the presence of active antigenic stimulation but form a part of the normal immune surveillance mechanism of mucosal tissue. We have shown that numbers of immune cells increase significantly in mares with recurrent acute endometritis and immunoglobulin production is known to be high (Asbury *et al.* 1980). However, these mares remain persistently infected. Although stimulation of local immunity has been shown to be important in elimination of infection in genitally normal mares (Widders *et al.* 1995) it is

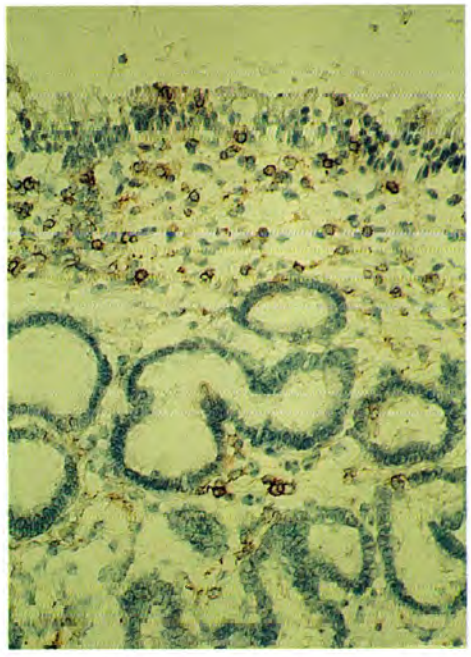

Fig 6: B cells in the endometrium of a mare with endometritis (avidin-biotin complex x25).

probable that the problem in recurrently infected mares is compounded by a defect in physical uterine clearance mechanisms through decreased myometrial contractility (Troedsson *et al.* 1993).

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Manufacturers addresses

[1]Miles Inc., Elkhart, Indiana, USA.
[2]British BioCell International, Cardiff, UK.
[3]Peterborough, Cambs, UK.

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Effect of repeated collection of multiple endometrial biopsy specimens on subsequent pregnancy in mares

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Summary: Endometrial biopsy specimens (4 or 5 on each occasion) were collected from 7 mares 2, 3, or 4 times over a 50-day period prior to breeding. Four of the collection days were within 6 days of breeding. Six of the 7 mares were diagnosed as pregnant by use of ultrasonography at day 14 after ovulation. This pregnancy rate was the same as that achieved by these mares when they were bred at estrus before the start of the study. It appeared that repeated collection of multiple endometrial biopsy specimens from genitally normal mares did not adversely affect pregnancy rate.

The collection of small pieces of endometrium via transcervical passage of endometrial biopsy forceps is commonly used in broodmare practice. A high correlation has been reported between inflammatory or chronic degenerative changes within the endometrium and subsequent fertility.[1,2] Accurate interpretation of these histologic changes permits evaluation of the ability of the endometrium to carry a foal to term. Although early work indicated that 1 biopsy specimen was adequate for the diagnosis and prognosis of uterine disease in mares,[3] more recent evidence suggests that although collection of a single biopsy specimen from the endometrium is generally adequate, it may not always provide results representative of the entire surface of the endometrium,[4,a] and in questionable cases, it has been suggested that 6 biopsy specimens should be collected from different sites within the uterus.[b] To our knowledge, the effect of obtaining multiple biopsy specimens on future pregnancy rates in mares has not been evaluated. In the study reported here, we were able to record pregnancy rates in mares that had been subjected to repeated collection of multiple biopsy specimens over a period of 50 days prior to breeding. Those specimens of endometrial tissue from mares at various stages of the estrous cycle had been used for endometrial cell culture procedures.[5] The purpose of the study reported here was to determine the effect of repeated collection of endometrial biopsy specimens on subsequent pregnancy in those mares.

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[a]Dybdal NO, Daels PF, Couto MA, et al. Investigation of the reliability of a single endometrial biopsy sample with a note on the correlation between uterine cysts on biopsy grade (abstr). *J Reprod Fertil Suppl* 1991;44:697.

[b]Kenney RM, University of Pennsylvania, Kennett Square, Pa: Personal communication, 1990.

Materials and Methods

Seven mixed-breed mares of light horse class, aged 4 to 13 years and weighing 450 to 550 kg, were used. The endometrium of all the mares was classified as category 1,[2] that is, no acute inflammatory or chronic degenerative changes were detected. Prior to biopsy, the mares were teased daily with a stallion to detect onset of estrus. The ovaries were examined daily through estrus by ultrasonography per rectum to monitor follicular growth and ovulation. When a 30-mm follicle was detected on the ovaries, the mares were artificially inseminated every other day until ovulation, with extended semen from a fertile stallion. To conform with the protocol of another experiment, embryos were recovered by flushing procedures performed on day 8 after ovulation.[6]

Over the next 2 months, endometrial biopsy specimens (4 or 5 on each occasion) were collected from each mare on 2, 3, or 4 occasions either on day 3 of estrus, day of ovulation, or days 3, 6, 10, or 14 of diestrus (Fig 1). Biopsy specimens were obtained from the mares in a clean, dust-free environment. Prior to biopsy, the mare's tail was placed in a plastic sleeve and tied up clear of the perineal region. The rectum was cleared of feces and the perineal region was scrubbed 4 times with povidone-iodine solution. The region was then dried with clean paper towels. The operator used a clean rectal sleeve lubricated with sterile jelly[c] to introduce the sterile, 2-cm jaw biopsy forceps[d] and

[c]H-R Lubricating Jelly, Carter Products, New York, NY.

[d]Pilling Manufacturing, Fort Washington, Pa.

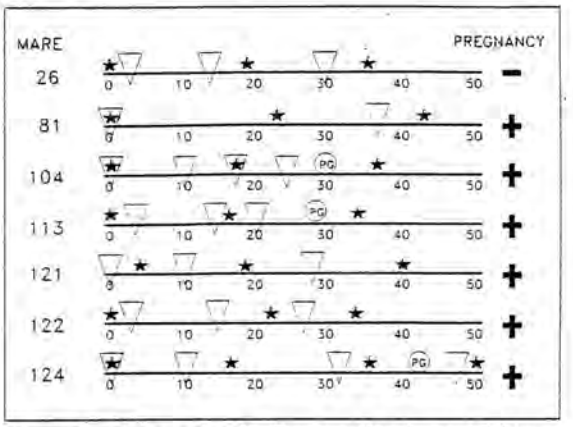

Figure 1—Time scale of collection of endometrial biopsy specimens (arrowheads) from mares relative to ovulation (stars) and effect on subsequent pregnancy (mares were bred during the estrus of the last depicted ovulation). Prostaglandin $F_{2\alpha}$ (PG) was administered to 3 of the mares during diestrus.

introducer rod (stainless steel cannula with a distal Teflon plug)[7] into the uterus. Between biopsies, the introducer rod was kept in place in the cervix so that the forceps could be reintroduced in a sterile fashion. On each occasion, one of the biopsy specimens was collected into Bouin's fixative and processed to detect any evidence of endometritis as a result of repeated biopsy. A section from one of the remaining biopsy specimens was stained with H&E and evaluated histologically. At the end of the study, the mares were bred as described previously, and pregnancy was diagnosed by use of ultrasonography on day 14 after ovulation.

Results

At the first breeding, embryos were collected from 6 of the 7 mares (no embryo was recovered from mare 121). Embryos were detected in 6 of the 7 mares after the second breeding (Fig 1). Histologic evaluation of 2 biopsy specimens (each approx 2 × 0.5 cm) on each occasion revealed the endometria of all mares remained in category 1[2] throughout the study. Mare 121 did not ovulate for 14 days after collection of a biopsy specimen (the third obtained from this mare) on day 6 of diestrus. This mare returned to estrus 4 days after collection of the biopsy specimen but developed an anovulatory follicle that persisted until a second smaller follicle developed and ovulated. One of the mares (mare 124) received prostaglandin $F_{2\alpha}$[e] on day 6 of diestrus, and then biopsy was done on day 3 of estrus. Two mares (104 and 113) received prostaglandin $_{2\alpha}$ to ensure their return to estrus because they were due to be bred at this estrus.

Discussion

Repeated collection of multiple biopsy specimens did not affect pregnancy rates in the 7 genitally normal mares in this study. Any procedure that involves manipulating the uterus or cervix of mares causes an acute generalized endometrial inflammatory response.[8] Histologic examination did not reveal neutrophil infiltration of the endometria even when the period between biopsy procedures was as short as 5 days. Accordingly, it appeared that the inflammatory reaction associated with collection of endometrial biopsy specimens remained localized and/or was of short duration. It should be emphasized, however, that in this study, attention was paid to hygiene to avoid unnecessary contamination of the uterus.

The condition of acute endometritis has been shown to be cytotoxic for early embryos, apparently because of inflammatory cells and their products.[9,10] In our study, biopsy specimens were collected 3 to 6 days prior to ovulation without adversely affecting conception. Furthermore, in another study in which 1 endometrial biopsy specimen was collected on day 6 after foaling, 12 of 13 mares became pregnant when bred at the first estrus after foaling.[11] The equine embryo reaches the uterus at 5 to 6 days after ovulation,[12,13] therefore in this study, the intervening interval of 8 to 12 days evidently allowed resolution of any uterine inflammation.

On the basis of our findings, frequent collection of multiple endometrial biopsy specimens did not reduce pregnancy rates in 7 genitally normal, fertile mares. It would appear, therefore, that hygienic biopsy of multiple specimens of endometrium would be unlikely to affect future conception in genitally normal mares. In a study by Hughes and Loy,[14] genitally normal mares conceived after repeated intrauterine infusion of bacteria. Our findings further emphasize the resilience of the normal endometrium to repeated insult. It would be useful to extend this study to subfertile mares with category 2 and 3 endometria.[2]

[e]Lutalyse, The Upjohn Co, Kalamazoo, Mich.

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Book Review
Equine Radiography

Equine Radiography by Morgan, Neves, and Baker provides a detailed, complete treatise on virtually all aspects of equine radiography. The book is divided into 2 sections: radiation physics and equine radiographic protocols.

The radiation physics section covers basic physics of x-ray production, x-ray tube function, circuitry, and accessories, such as collimation and the use of grids. There are excellent diagrams and pictures to augment the text. A large section is devoted to screens and all aspects of film type, use, and marking. Principles of radiographic quality, errors, technique charts, and radiation safety, are covered, including preparation of horses. This is the most complete and informative section available for horses. The completeness of this section makes it a unique contribution to the equine imaging literature.

The second section, equine radiographic protocols, covers all aspects of positioning and technique and uses a very organized unique approach. Each subsection provides a short text explaining the site to be studied, which includes animal preparation and positioning. Specifics for technique for the particular study are given in table form. Suggested film screen combinations are given, and physical values of the exposure are included. Each subsection has ample, large, good-quality pictures and diagrams. Examples of pathologic features are included where appropriate in explaining the value of the various views.

This text completely covers the subject of equine radiography in a unique, very well-illustrated and organized format. This book would be of value to every veterinary student and all equine practitioners. It is reasonably priced for the information it provides.—*[Equine Radiography. By J. P. Morgan, J. Neves, and T. Baker. 384 pages; illustrated. Iowa State University Press, 2121 S State Ave, Ames, IA 50010. 1991. Price $89.95.]*

NORMAN W. RANTANEN

Influence of administration of ovarian steroids on the function of neutrophils isolated from the blood and uterus of ovariectomized mares

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ABSTRACT

The function of blood and uterine luminal neutrophils from ovariectomized mares treated with ovarian steroids was investigated 18 h after intrauterine infusion of 1×10^9 *Streptococcus zooepidemicus*. Random migration of blood neutrophils under agarose was reduced by treatment with progesterone compared with that of neutrophils from oestradiol-treated and control mares. In-vitro addition of progesterone to blood neutrophils from acyclic ponies also reduced migration. Uterine neutrophils did not migrate under agarose which was probably an effect of bacterial phagocytosis. Hormone treatment had little effect on phagocytosis of yeast blastospores by blood neutrophils. Phagocytosis by uterine neutrophils from oestradiol-treated and control mares was significantly better than that by blood neutrophils. In progesterone-treated mares, however, phagocytosis by uterine neutrophils was significantly lower than that in the other two treatment groups and was similar to that measured in blood neutrophils.

The results indicate a marked effect of progesterone in reducing both migration of blood neutrophils and phagocytosis by uterine neutrophils.

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INTRODUCTION

The uterus of the mare is highly susceptible to establishment of infection by invading micro-organisms when under the influence of increased concentrations of progesterone in the blood (Ganjam, McLeod, Klesius *et al.* 1982). The increased dependence on immunoglobulin G (IgG) and IgM in the uterus of the mare compared with that of other mucosae, such as the intestine (Widders, Stokes, David & Bourne, 1984), suggests that phagocytic cells play a major role in combating infection. The main phagocyte involved in cellular defence within the uterus is the neutrophil, and by 2 h after introduction of infection neutrophilic infiltration of the uterus is intense (Williamson, Dunning, O'Connor & Penhale, 1983). In order to reach the uterine lumen, neutrophils must be able to respond to chemotactic stimuli and migrate from the uterine vasculature through the endometrium. In-vitro experiments using bovine blood neutrophils have shown that ovarian steroids may influence neutrophil locomotion. High plasma concentrations of either oestradiol-17β or progesterone in cyclic cows enhanced random migration of blood neutrophils *in vitro* (Roth, Kaeberle & Hsu, 1982). Furthermore, workers who used chemiluminescence to measure phagocytosis by blood neutrophils showed that ovarian hormones may modulate the phagocytic capacity of these cells (Ganjam *et al.* 1982). However, there is no information on the effect of ovarian steroids on the function of uterine luminal neutrophils. The purpose of the present study was to investigate the effect of physiological concentrations of ovarian steroids on phagocytosis and migration by blood- and uterine-derived neutrophils; in particular, any deficiencies in function which might be associated with increased plasma concentrations of progesterone.

MATERIALS AND METHODS

Each of four ovariectomized pony mares which were judged to have normal endometria by histological examination of biopsies were treated daily for 7 days with i.m. injections (4 ml) of progesterone (100 mg), oestradiol benzoate (1 mg) or vehicle (arachis oil;

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RESULTS

Hormone treatment did not alter the total counts of white blood cells or percentage of neutrophils in whole blood. By 18 h after bacterial infusion, no bacteria were isolated from washings of any of the mares receiving oestradiol. Only one control mare had a light growth of *S. zooepidemicus*, but all progesterone-treated mares had heavy growths of *S. zooepidemicus* in washings cultured on blood agar plates overnight at 37 °C.

Neutrophil migration

The overall coefficient of variation for random migration between wells and plates varied between 10 and 14% for 21 replicate wells on seven different plates using neutrophils from five ponies. Blood neutrophils from mares treated with progesterone moved less far ($P<0.05$) on random migration plates than those from mares treated with oestradiol or from control mares (Table 1).

TABLE 1. Mean distance migrated under agarose by neutrophils from ovariectomized mares treated daily for 7 days with oestradiol benzoate, progesterone or vehicle (arachis oil). Values are means for five replicate plates

	Mean distance migrated (mm)		
	Oestradiol	Progesterone	Arachis Oil
Assay No.			
1	1·18[b]	0·65[c]	1·04[a] 1·25[d]
2	1·33[a]	0·97[b]	1·35[c] 1·13[d]
3	1·33[b]	1·01[c] 1·23[d]	1·36[a]
4	0·89[c] 1·14[d]	0·45[a]	0·84[b]
5	0·56[a] 0·96[d]	0·38[b]	0·67[c]
6	1·50[c]	1·35[a] 0·77[d]	1·39[b]

Superscript letters denote individual mares.

When oestradiol-17β was added to cells *in vitro*, random migration was significantly ($P<0.001$) depressed at 5 pg/ml and then returned to control levels when 25 pg oestradiol/ml were added. Addition of 100 pg oestradiol/ml again resulted in depression ($P<0.05$) of migration (Fig. 1). With addition of progesterone, migration was significantly depressed at all concentrations ($P<0.001$).

Assays to study migration of uterine neutrophils under agarose proved unsuccessful. In an attempt to explain this result, blood neutrophils were incubated with *S. zooepidemicus* to monitor the effect of activation and phagocytosis on migration. Incubation of neutrophils at 37 °C reduced migration (0·71 ± 0·04 mm) compared with neutrophils remaining at room temperature (1·11 ± 0·07 mm). Neutrophils showed a progressive decrease in random migration after phagocytosing increasing numbers of bacteria (Table 2). Migration was significantly ($P<0.01$) reduced when there were on average more than 0·39 bacteria per neutrophil with 12·5% of neutrophils containing bacteria.

Yeast phagocytosis assay

Only three of the four mares treated with oestradiol yielded adequate numbers of uterine neutrophils to perform the phagocytosis assay. All three treatment groups had similar rates of phagocytosis for neutrophils isolated from blood (Table 3), with the only significant ($P<0.05$) difference occurring between progesterone-treated and control mares in the number of neutrophils containing no yeast blastospores. Uterine neutrophils were significantly better than blood neutrophils at ingesting yeast blastospores in control ($P<0.01$) and oestradiol-treated ($P<0.05$) mares. In progesterone-treated mares, however, uterine neutrophils ingested significantly ($P<0.01$) fewer blastospores than uterine neutrophils of the other two treatment groups and had a similar phagocytic index to that of blood neutrophils.

DISCUSSION

Migration is an important functional characteristic of neutrophils in response to an inflammatory stimulus such as bacterial invasion. Administration of progesterone and oestradiol to mares to achieve plasma concentrations consistent with dioestrus and oestrus respectively, caused a reduction in neutrophil migration in progesterone-treated mares. In contrast, random migration of neutrophils from cyclic cows with high physiological concentrations of progesterone or oestradiol was enhanced (Roth, Kaeberle, Appell & Nachreiner, 1983). However, the control cows in the latter study still had low circulating concentrations of hormones, whereas the control mares in the present study were ovariectomized, and this may explain the apparent discrepancy. In the present study, in-vitro addition of progesterone to neutrophils from acyclic ponies resulted in a similar depression in migration to that following in-vivo administration of progesterone. When low concentrations of oestradiol (5 pg/ml), such as might occur in the dioestrous cow or mare, were added *in vitro*, random migration was depressed compared with that after addition of higher concentrations (25 pg/ml) which might occur during oestrus. Thus when oestrous concentrations are

TABLE 3. In-vitro phagocytosis of yeast blastospores by neutrophils from ovariectomized mares treated with progesterone, oestradiol benzoate or vehicle (arachis oil). Values are means ± S.E.M; n = number of mares

		% blood neutrophils ingesting 0 or ⩾ 2 yeast blastospores			% uterine neutrophils ingesting 0 or ⩾ 2 yeast blastospores	
	n	0	⩾ 2	n	0	⩾ 2
Hormone treatment						
Vehicle	4	54 ± 3·2	30 ± 4·2	4	28 ± 5·4	58 ± 4·7
Oestradiol benzoate	4	57 ± 3·6	29 ± 4·4	3	40 ± 5·0	45 ± 2·6
Progesterone	4	64 ± 2·6	20 ± 1·8	4	65 ± 2·6	17 ± 1·7

ovarian steroids over a period of 5 weeks which may have had a direct effect on blood cells.

Uterine-derived neutrophils from control and oestradiol-treated mares were better at phagocytosis than blood neutrophils. It is possible that this increased phagocytosis was because the exudate neutrophils were already activated whereas those from the blood were presumably unstimulated. Treatment with progesterone profoundly reduced the phagocytic activity of uterine neutrophils to a level similar to that of blood neutrophils. The similarity between phagocytic activity in blood and uterine neutrophils collected from genitally normal mares in mid-dioestrus has been reported previously (Cheung, Liu, Walsh & Miller, 1985). It is not known whether this apparent deficiency in uterine neutrophil activity during periods of high concentrations of plasma progesterone is a primary cause of persistence of infection in mares. It may be that neutrophil activity is affected adversely secondary to persistence and multiplication of the bacteria resulting from a primary predisposing factor, such as a change in morphology of endometrial cells, which may influence bacterial adhesion during periods of circulating progesterone dominance.

Although the suppression of neutrophil activity by progesterone has been widely reported in various species (Hawk, Turner & Sykes, 1960; Ganjam, *et al.* 1982; Matsuda, Okuda, Fukui & Kamata, 1985), there are marked species differences in the uterine inflammatory response under the influence of oestrogens (Brinsfield, Hawk & Leffel, 1963). In the present study, administration of oestradiol had little effect on neutrophil activity but progesterone suppressed migration and possibly phagocytosis by blood and uterine neutrophils respectively. The mechanism of suppression is not known but cortisol and related steroids also suppress neutrophil migration and phagocytosis. This is presumed to be a direct antagonistic effect of corticosteroids on the cell (Ward, 1966; Majeski & Alexander, 1976) and it is possible that, as suggested in the cow, progesterone may influence neutrophil function through a similar mechanism (Roth *et al.* 1982). It is not known whether these effects are mediated by receptors, as receptors for sex hormones have not yet been detected on neutrophils, although oestradiol-17β is firmly bound to neutrophils during phagocytosis (Klebanoff, 1977) and non-specific binding of progesterone and other steroids by lymphocytes is appreciable (Munck & Brinck-Johnson, 1968). Alternatively, neutrophils are capable of metabolizing progesterone (Scully, Ferguson & Grant, 1982) and thus it is possible that a metabolite may be the active component rather than the parent compound. Steroids might also exert their action by membrane effects, as their lipophilic properties correspond approximately to their suppressive effect (Staples, Binns & Heap, 1983). Alternatively, the hormone may interact with lymphocytes present in the leucocyte preparation. Lymphocytes have been shown to be influenced by ovarian steroids (Clemens, Siiteri & Stites, 1979) and can produce factors which inhibit neutrophil function (Rocklin, 1974; Goetzl, 1978).

Thus the results of the present study, which showed a decrease in neutrophil function *in vitro*, may partially explain the increased susceptibility to uterine infection during periods of progesterone domination. In another study, however, which investigated the speed of mobilization of neutrophils into the uterine lumen, there was no significant difference in the first 6 h after induction of uterine inflammation between ovariectomized mares treated with progesterone, oestrogen or vehicle (Watson, Stokes, David & Bourne, 1987*c*). More detailed studies are therefore required to understand the complex cellular interactions with ovarian hormones and their effect on disease resistance in the mare.

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Effect of ovarian hormones on promotion of bactericidal activity by uterine secretions of ovariectomized mares

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Summary. The bactericidal and phagocytic activities of blood neutrophils suspended in uterine washings and the mobilization of neutrophils into the uterine lumen were studied in ovariectomized mares receiving oestradiol benzoate (N = 4), progesterone (N = 4) or oily vehicle (N = 4). Uterine lavage was performed sequentially up to 144 h after induction of endometritis by intrauterine infusion of glycogen (1%). There was no significant difference between the 3 groups in speed of mobilization of neutrophils into the uterus in the first 6 h after infusion but there were significantly more uterine luminal neutrophils in progesterone-treated than in oestradiol-treated mares by 24 h after infusion ($P < 0{\cdot}01$). Uterine washings collected from progesterone-treated mares at 0, 24 and 144 h were significantly worse at promoting bactericidal activity by neutrophils than washings from oestradiol-treated and control mares ($P < 0{\cdot}001$). In oestrogen-treated and control mares bactericidal activity had increased by 144 h but in progesterone-treated mares bactericidal activity remained low. Neither treatment nor time affected the ability of washings to opsonize yeast blastospores. Elevated concentrations of progesterone in plasma were therefore associated with decreased bactericidal activity of neutrophils suspended in uterine washings but the generation of C_{3b} in washings did not appear to be affected by hormone treatment.

Introduction

Uterine infection in mares is one of the major causes of failure of conception (Bain, 1966) and the bacteria most commonly implicated in this subfertility are β-haemolytic streptococci (Hughes & Loy, 1975). In many species the uterus is more susceptible to uterine infection in the progesterone-dominated luteal phase than in the oestrogen-dominated follicular phase of the oestrous cycle. This deficiency may relate to cellular factors within the uterus, such as mobilization of phagocytes and their efficiency at phagocytosis and intracellular killing, or to humoral factors which may be immunologically specific or non-specific.

The components in uterine secretions which promote bacterial killing have not been studied in detail. When present in sufficiently high concentrations, antibody directed against specific antigenic determinants on bacterial cell walls will opsonize the bacteria and aid phagocytosis by neutrophils (Stossel, 1974). This process is greatly enhanced, however, by activation of the classical or alternative complement pathways. Deposition of one of the components of the complement pathway, C_{3b}, has been shown to be necessary for phagocytosis of *Streptococcus zooepidemicus* by equine neutrophils (Asbury *et al.*, 1984). Direct activation of the alternative pathway by *S. zooepidemicus* is suggested to be of little importance (Asbury *et al.*, 1984), although another group C streptococcus, *S. equi*, is known to be a potent activator of the alternative pathway of complement (Mukhtar & Timoney, 1985).

In the mare the clinical response to intrauterine infusion of bacteria was thought to be similar in oestrus and dioestrus (Peterson *et al.*, 1969; Hughes & Loy, 1975). More recent work has shown that infection persisted in progesterone-treated ovariectomized mares, suggesting an hormonal

influence on the ability to eliminate bacteria from the uterus (Ganjam *et al.*, 1982). In cattle the ability of uterine secretions to promote bactericidal activity is reduced during the luteal phase of the oestrous cycle (Watson, 1985). However, conflicting results have been obtained for mares when different methods have been used to assess phagocytosis and bactericidal activity of neutrophils suspended in uterine washings (Blue *et al.*, 1982; Brown *et al.*, 1985).

The present study investigated the efficacy of killing of *Streptococcus zooepidemicus* when neutrophils were suspended in uterine washings collected from hormone-treated ovariectomized mares after induction of an immunologically non-specific endometritis. Secondly, the efficiency of opsonization of yeast blastospores by uterine washings was studied to monitor any changes in complement-mediated opsonization which might be induced by ovarian steroids.

Materials and Methods

Animals and experimental design. Each of 4 ovariectomized Pony mares weighing 250–300 kg was treated on 3 separate occasions for 14 days with daily i.m. injections (4 ml) of oestradiol benzoate (0·25 mg/ml), progesterone (25 mg/ml) or the oily vehicle, arachis oil (Intervet, Cambridge, U.K.). On each occasion ($n = 3$) every mare ($N = 4$) received different hormone treatments. This treatment regimen has been shown to induce changes in plasma hormones and in the histological morphology of the endometrium consistent with oestrus, dioestrus and anoestrus respectively (E. D. Watson, unpublished data).

On Day 8 of hormone treatment, uterine washings were collected via a Foley catheter (24 FG, 30 ml cuff) by flushing the uterus with 40 ml sterile phosphate-buffered saline (PBS; 0·1 M, pH 7·0) (Watson *et al.*, 1987). Oyster glycogen (1%) in 50 ml sterile PBS was infused into the uterus before withdrawal of the catheter to induce an acute inflammatory response. This method of inducing acute endometritis has previously been described for cattle (Anderson *et al.*, 1984; Watson, 1985) and mares (Watson *et al.*, 1987). The washing procedure was repeated at 1, 3, 6, 24 and 144 h after infusion.

Treatment of washings. Uterine washings were kept on ice until centrifuged at 125 *g* for 10 min. Cells in the pellet were counted using a haemocytometer. Cell viability was determined by addition of trypan blue dye (0·1%; 1:1, v/v) and calculation of the percentage of cells which excluded the dye. Differential cell counts were performed using a cytospin preparation (Dulin *et al.*, 1982) stained with Giemsa dye (Diff-Quick, American Hospital Supplies, Compton, Berkshire, U.K.). The supernatant was further centrifuged at 10 000 g for 30 min, decanted into aliquants and frozen at −70°C. To detect the presence of aerobic bacteria in washings a drop of fluid from each flushing was streaked onto a blood agar plate and incubated at 37°C for 48 h.

Bactericidal assay. This assay, along with isolation of blood neutrophils, was performed as described by Watson *et al.* (1987) using 0·1 ml *Streptococcus zooepidemicus* (3×10^6/ml), 0·2 ml neutrophils (1×10^7 ml) collected from one gelding and 0·3 ml uterine washing. All washings were sterilized by filtration before use (0·45 μm Millipore; Flow Laboratories, Irvine, U.K.). The tubes were prepared in duplicate and were rolled at 37°C for 2 h. The numbers of viable colony forming units (c.f.u.) which remained at the end of the incubation were assessed by spotting 0·02 ml drops of 3 × 10-fold dilutions in sterile saline onto blood agar plates. The survival of bacteria was calculated by: (no. of c.f.u. obtained with test uterine washings/no. of c.f.u. in duplicate tubes in which the neutrophils were replaced by 0·2 ml Hank's Balanced Salt Solution (HBSS) and the uterine washings by 1% horse serum) × 100%.

Control tubes were those in which uterine washings or neutrophils were replaced by PBS.

Yeast phagocytosis assay. This assay was performed as described by Soothill & Harvey (1976) with minor modifications. Blood neutrophils were isolated as described by Watson *et al.* (1987) and 1×10^8 heat killed yeast blastospores/ml (0·2 ml) were added to 0·1 ml washing and 0·1 ml neutrophils (1×10^7/ml). Tubes were rolled for 45 min at 37°C and then centrifuged at 50 *g* for 5 min. The sediment was resuspended in saline (0·9% (w/v) NaCl, 1·5 ml) and a cytospin preparation was stained with Giemsa. The number of yeast blastospores ingested by 100 neutrophils was counted and the phagocytotic index was expressed as blastospores/neutrophil.

Total protein assay. Total protein was measured in washings using a Lancer Microprotein Rapid Stat Diagnostic Kit (Clandon Scientific Ltd., Aldershot, U.K.). The method was based on formation of a dye–protein complex with Coomassie Brilliant Blue dye. Protein concentrations were assessed colorimetrically.

Statistical methods. The effects of horse and time on the variates were analysed by a blocked analysis of variance (with horse as the blocking factor) using a computer statistical package (Genstat, Rothamsted, U.K.). Relationships between measurements were analysed by the Pearson correlation coefficient test. Results were considered significant when $P < 0·05$. All means are quoted ± s.e.m.

Results

All washings were culturally negative for bacteria except at 144 h for 2 mares treated with progesterone. Bacteria isolated from 1 of these mares were *Streptococcus zooepidemicus* and from the other *Staphylococcus aureus*.

In all statistical analyses the effect of horse was not significant.

Infusion of glycogen resulted in a large influx of neutrophils into the uterus by 24 h regardless of hormone treatment (Fig. 1). Progesterone treatment significantly increased numbers of intrauterine neutrophils at 24 and 144 h after infusion compared with the other two groups. The number of neutrophils in washings collected at 1, 3 and 6 h after infusion of glycogen increased significantly with time ($P < 0{\cdot}01$) but there was no significant difference among the three treatment groups in speed of mobilization of neutrophils into the uterine lumen (Table 1). When bacteria were

Table 1. Rate of mobilization of neutrophils into the uterine lumen of ovariectomized mares treated with ovarian steroids

Hormone treatment	Time after infusion (h)	Mean (±s.e.m.) no. of viable neutrophils in washing (log_{10})
Oestrogen	1	2·0 ± 0·85
	3	5·9 ± 0·40
	6	7·5 ± 0·41
Progesterone	1	4·3 ± 0·30
	3	5·7 ± 0·25
	6	8·2 ± 0·11
Control	1	2·8 ± 1·65
	3	6·0 ± 0·40
	6	7·7 ± 0·21

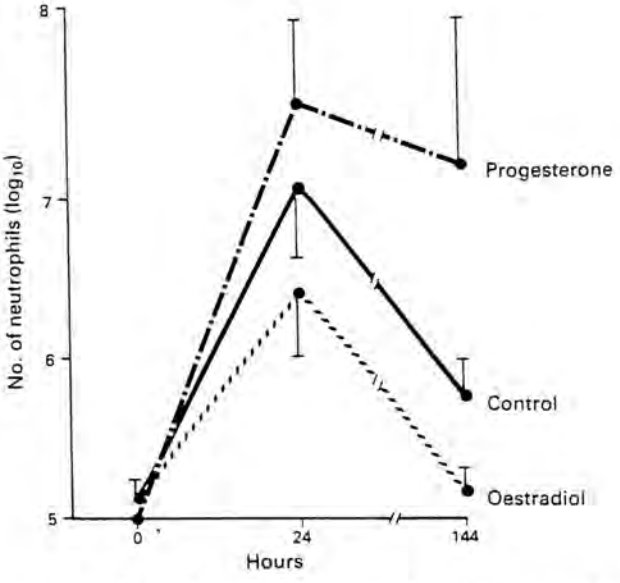

Fig. 1. Mean (±s.e.m.) numbers of viable neutrophils in uterine washings collected after infusion of glycogen from mares treated with oestrogen, progesterone or arachis oil. At 24 h after infusion, neutrophil numbers were higher in washings from progesterone-treated than oestrogen-treated mares ($P < 0{\cdot}01$). By 144 h, progesterone-treated mares had more intraluminal neutrophils than either of the other groups ($P < 0{\cdot}001$).

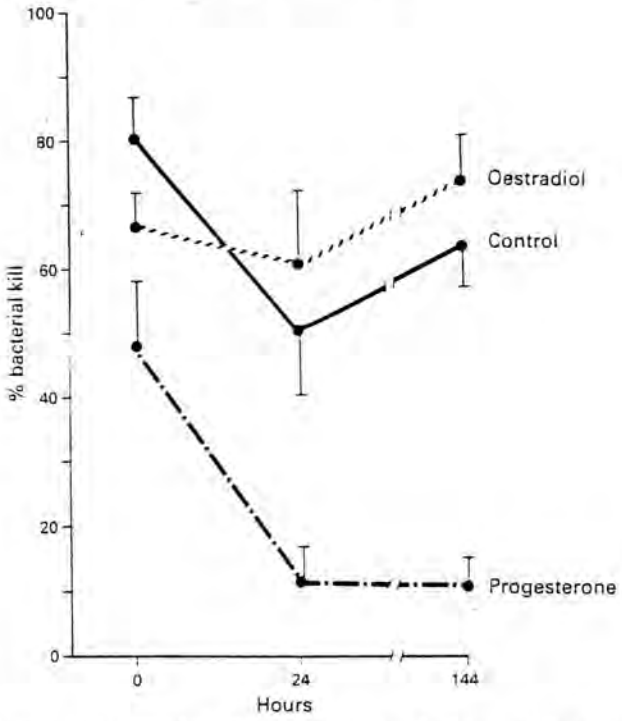

Fig. 2. Mean (±s.e.m.) percentage kill of *S. zooepidemicus* by neutrophils suspended in uterine washings collected after infusion of glycogen from mares treated with oestrogen, progesterone or arachis oil. Overall, both time after infusion ($P < 0{\cdot}01$) and hormone treatment ($P < 0{\cdot}001$) had a significant effect on bactericidal activity of neutrophils. Before infusion, washings from progesterone-treated mares were significantly worse at promoting bactericidal activity than those from oestrogen-treated ($P < 0{\cdot}02$) or control mares ($P < 0{\cdot}001$). At both 24 and 144 h after infusion, bacterial kill was significantly lower ($P < 0{\cdot}001$) with washings from progesterone-treated mares than from the other two groups.

Table 2. Phagocytosis of yeast opsonized by uterine washings from ovariectomized mares treated with ovarian steroids

Time of collection of washings after infusion glycogen (h)	Number of yeast particles/neutrophil		
	Oestrogen	Progesterone	Controls
-0	0·800	0·850	0·600
24	0·975	0·825	0·750
48	1·300	0·975	1·150

Hormone × time s.e.d. = 0·369.

incubated with neutrophils in the absence of washings, 6% of bacteria were killed. Incubation of bacteria with 6 different uterine washings in the presence or absence of neutrophils resulted in a decrease in mean bacterial kill from 53 to 16% ($P < 0{\cdot}02$). Neutrophils suspended in uterine washings from mares treated with progesterone were significantly worse at killing bacteria than those from oestrogen-treated or control mares (Fig. 2).

The number of yeast blastospores ingested by neutrophils after opsonization in uterine washings was significantly greater ($P < 0{\cdot}001$) than after substitution of washings by PBS ($0{\cdot}23 \pm 0{\cdot}05$/neutrophil). Neither treatment nor time of collection of washings influenced the ability of washings to opsonize yeast particles (Table 2).

Before infusion of glycogen, there was no significant difference in protein concentrations between mares treated with progesterone (100 ± 60 μg/ml), oestradiol (51 ± 22 μg/ml) or controls (40 ± 19 μg/ml). Overall, however, mean protein concentrations were higher ($P < 0{\cdot}001$) in uterine washings from progesterone-treated mares (587 ± 196 μg/ml) than in oestrogen-treated mares (63 ± 15 μg/ml). Protein concentrations in control mares (207 ± 134 μg/ml) were not statistically different from those in progesterone-treated mares.

The percentage kill of bacteria *in vitro* was inversely related both to the protein concentration of the uterine washings ($r = -0{\cdot}502$; $P < 0{\cdot}01$) and to the number of neutrophils originally present in the washing ($r = -0{\cdot}484$; $P < 0{\cdot}01$). The number of neutrophils present in washings was directly correlated with the protein concentration of the washing ($r = 0{\cdot}834$; $P < 0{\cdot}001$). Opsonization of yeast blastospores by uterine washings was not related to number of neutrophils originally present in the washing, percentage bacterial kill or protein concentration of the washings.

Discussion

Opsonization is required for phagocytosis of *Streptococcus zooepidemicus* by horse neutrophils (Asbury *et al.*, 1982). Little is known of the process of opsonization of bacteria by factors present in uterine secretions, but since inactivation of complement in uterine secretions almost abolishes phagocytosis it would appear that deposition of C_{3b} on the bacterial cell wall may have an important role (Asbury *et al.*, 1984). In the present study phagoctyosis of yeast blastospores was used to assess changes in the deposition of C_3 fragments in uterine secretions from mares treated with oestradiol or progesterone. In the absence of specific antibody, yeasts will activate complement via the alternative complement pathway (Soothill & Harvey, 1976). It has been suggested, however, that in some cases antibody may be present which permits the classical pathway of complement to contribute to C_{3b} generation (Turner *et al.*, 1985). In uterine washings the relative importance of this contribution remains to be determined. Impaired phagocytosis of yeast blastospores after opsonization with human serum has been used to detect defects in the alternative pathway of complement activation and deposition of C_3 fragments (Soothill & Harvey, 1976; Turner *et al.*, 1986). Uterine washings were capable of significantly enhancing phagocytosis compared with PBS and C_{3b} could therefore be generated in uterine secretions. Other workers have suggested that although *S. zooepidemicus* did not seem to activate the alternative pathway of complement directly, this pathway is likely to play a role in opsonization of gram-negative bacteria (Asbury *et al.*, 1984).

The results of the present study showed that neither hormone treatment nor induction of acute endometritis affected the generation and deposition of complement fragments in washings on to yeast blastospores. This finding contrasted with results from cattle and rabbits; washings from luteal-phase animals contained a seromucoid component which inhibited phagocytosis of starch particles (Killingbeck & Lamming, 1963).

The killing of bacteria by neutrophils in the presence of uterine washings collected before infusion of glycogen was high in oestrogen-treated and control mares but was significantly depressed in mares treated with progesterone. Brown *et al.* (1985) used chemiluminescence to study bacterial phagocytosis and reported very low light emission when bacteria were opsonized with washings from uninfected mares in oestrus and dioestrus. This apparent contradiction may be due to the different techniques used to study opsonization. In the present study non-specific bactericidal substances present in washings may have contributed to the results.

After induction of acute endometritis, bacterial kill by neutrophils decreased markedly when suspended in washings from progesterone-treated mares and remained low until the end of the

experiment when 2 of the 4 progesterone-treated mares succumbed to uterine infection. The failure to demonstrate any suppression of yeast phagocytosis by neutrophils suspended in washings from progesterone-treated mares would suggest that it is unlikely that factors present in washings had a direct effect on neutrophil phagocytosis. However, the uterine washings may have modified the bactericidal activity of neutrophils in addition to their role as bacterial opsonins.

In uterine washings from normal mares, IgG levels relative to total protein were apparently depressed during dioestrus (Widders *et al.*, 1985). The reduced availability of this immunoglobulin when plasma concentrations of progesterone were high may have contributed to the decreased opsonization by washings. Alternatively, the large numbers of neutrophils in washings from progesterone treated mares may have caused breakdown of immunoglobulins by release of neutrophil proteases (Waller, 1974) which would explain the significant negative correlation between numbers of neutrophils present in washings and opsonizing ability of the washings.

Uterine neutrophils may have a phagocytic function which is greater than or similar to that of blood neutrophils depending on the method of stimulating an acute endometritis (Targowski *et al.*, 1985). It is likely that uterine neutrophils collected in the presence of bacterial infection are already activated whereas the circulating neutrophils used in this study were presumably unstimulated at the time of collection. It would therefore be interesting to extend the present study to investigate the interaction between uterine neutrophils and washings.

Killing of bacteria by neutrophils was inversely related to the protein concentration of the opsonizing washing. However, when uterine fluids were diluted so that the protein concentration was equivalent in all samples, bacterial killing did not change significantly and remained significantly correlated with washings which were not corrected for protein concentration ($n = 11$; $r = 0{\cdot}688$; $P < 0{\cdot}02$).

Previous work showed that only during dioestrus did uterine fluid from mares inhibit growth of *Streptococcus zooepidemicus* in the absence of neutrophils (Strzemienski *et al.*, 1984). In the present study there was little evidence of significant bacterial killing in the absence of neutrophils, but further work is needed to elucidate the role of bactericidal proteins in uterine washings from hormone-treated ovariectomized mares. Bactericidal proteins such as lactoferrin (Dixon & Gibbons, 1979), lysozyme (Roberts *et al.*, 1976) and peroxidase (Linford, 1974) have been isolated from uterine fluid of other species. Peroxidase has been measured in uterine washings from mares but was present in very low concentrations in uninfected uteri and was not correlated with stage of oestrous cycle (Blue *et al.*, 1982). In the present study the bacterial kill was inversely related to the number of neutrophils originally present in the washing. The increased concentrations of peroxidase which are associated with the presence of high numbers of neutrophils (Linford, 1974) must therefore have had a negligible effect on bacterial killing due to other overriding factors present in the progesterone-treated mares.

In the present study hormone treatment did not affect speed of mobilization of neutrophils in the first 6 h after infusion, although in other species high plasma concentrations of progesterone have been shown to delay intrauterine migration of neutrophils (Broome *et al.*, 1960; Hawk *et al.*, 1961). Delayed neutrophil mobilization in the mare is therefore not the explanation of the increased susceptibility to infection in the progesterone-treated mares and, by 24 h after infusion, the progesterone-treated mares had significantly more uterine luminal neutrophils than did the other groups.

In conclusion, progesterone administration did not affect mobilization of neutrophils into the uterine lumen or the generation of C_{3b} in uterine washings. However, the ability of washings to promote bactericidal activity by neutrophils was significantly reduced in these mares, which may be an important factor in increasing the susceptibility of dioestrous mares to intrauterine infection.

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Effect of exogenous ovarian steroids on the uterine luminal prostaglandins in ovariectomised mares with experimental endometritis

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Prostaglandins (PGs) F and E_2 were measured in lavage fluid from the uterus of ovariectomised mares after experimental induction of uterine inflammation. Treatment with progesterone alone, or progesterone followed by oestradiol, significantly increased the concentrations of these PGs in the lavage compared with mares treated with oestradiol or control mares. Ovarian steroids, therefore, influenced uterine PG synthesis in response to an inflammatory stimulus. To determine whether the uterine lavage procedure might contribute to the concentrations of prostaglandins in the lavage, the procedure was also performed on six intact mares. With the exception of washings obtained at luteolysis, uterine concentrations of PGF (measured as the plasma metabolite 15-keto-13,14-dihydro $PGF_{2\alpha}$) had returned to prewashing levels within 30 minutes of the start of uterine lavage. Lavage was therefore unlikely to have influenced the concentrations of prostaglandins in the lavage fluid.

ACUTE inflammation of tissues results in synthesis of prostaglandins (Weissman 1980). Measurement of prostaglandin (PG) concentrations in lavage fluids after intrauterine infusion of an irritant has shown that the uterus of the mare releases large amounts of PGF and PGE_2 in response to an inflammatory stimulus (Watson et al 1987a). PGF is thought to be the naturally occurring luteolysin in the mare (Douglas and Ginther 1976) and the development of acute bacterial endometritis has led to premature luteolysis (Allen and Hadley 1974). Synthesis of PGF by the endometrium of the mare is influenced in vitro by ovarian steroids and the greatest concentrations are produced when oestradiol acts on progesterone-primed endometrium (Vernon et al 1981). Furthermore, the response of the ovine uterus in vivo to a range of physiological and pathological stimuli measured by synthesis of $PGF_{2\alpha}$ was dependent on the circulating concentrations of ovarian steroids at the time of the insult (Roberts et al 1975).

There is no information on concentrations of PGE_2 in endometrial tissue or in uterine luminal secretions of mares in different stages of the cycle or during pregnancy. In sheep and cattle, however, an anti-luteolytic role has been suggested for PGE_2 (Speroff and Ramwell 1970, Henderson et al 1977, Ellinwood et al 1979, Gimenez and Henricks 1983).

In mares, concentrations of PGE_2 and PGF in uterine lavage increased after induction of acute inflammation in two dioestrous mares, but there was a smaller response in a mare sampled during oestrus (Watson et al 1987a). The present study investigated further the effect of ovarian steroids on synthesis of PGF and PGE_2 by the uterus before and after induction of endometritis in ovariectomised mares. The effect of the washing procedure on production of uterine prostaglandins was also determined.

Materials and methods

Experimental animals

Using a blocked experimental design, each of four ovariectomised mares weighing 250 to 300 kg was treated daily for eight days with intramuscular injections (4 ml) of either oestradiol benzoate (1 mg), progesterone (100 mg) or the oily vehicle (arachis oil). Each of these treatments had been shown to produce changes in the endometrium and in plasma concentrations of oestradiol and progesterone consistent with oestrus and dioestrus respectively (Watson 1986). A fourth treatment consisted of injections of progesterone (100 mg) for five days followed by oestradiol benzoate (1 mg) for three days. Each of the four treatments was administered sequentially to each mare with an interval of at least 14 days between treatments. On day 7 of injections, uterine washings were collected and the mares then received an intrauterine infusion (50 ml) of 1 per cent oyster glycogen to induce endometritis (Watson et al 1987a). Further

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washings were obtained at six and 24 hours after infusion.

Uterine washing technique

After cleansing the vulva and perineal region, 40 ml of sterile phosphate buffered saline (pH 7·0) was infused into the uterus through a 24 FG Foley catheter. The fluid was aspirated and reintroduced several times with a catheter-tip syringe before collection. The washings were placed on ice until centrifugation at 10,000 *g* for 30 minutes at 4°C. The supernatant was stored at −70°C until assay. Samples from the washings were cultured on blood agar plates and incubated aerobically for 48 hours at 37°C to detect the presence of bacteria.

Effect of washing procedure on prostaglandin production

A single uterine washing (40 ml sterile phosphate buffered saline) was collected from six genitally normal mares (250 to 300 kg). All mares had a palpably normal genital tract with no histological evidence of endometritis in endometrial biopsies. Two of the mares were in mid-dioestrus, two were in winter anoestrus, one mare was in oestrus and one was undergoing luteolysis. Samples of jugular venous blood were collected into heparinised vacutainers (Becton-Dickinson) before, and then at intervals up to 30 minutes after the start of the washing procedure (which lasted less than three minutes). Blood samples were then collected periodically for the next six to 48 hours. The samples were kept on ice until centrifugation at 2000 *g* for 10 minutes at 4°C. Plasma was aspirated and stored at −20°C until assayed for concentrations of the $PGF_{2\alpha}$ metabolite, 15-keto-13,14-dihydro $PGF_{2\alpha}$ (PGFM).

PGF and PGE_2 radioimmunoassay

Concentrations of immunoreactive PGF and PGE_2 were measured in the uterine lavage by radioimmunoassay without prior extraction (Watson et al 1987a). The main cross reactions of the $PGF_{2\alpha}$ antiserum (over 0·5 per cent) were $PGF_{1\alpha}$ 75 per cent and $PGF_{2\beta}$ 1·5 per cent and of the PGE_2 antiserum was PGE_1 3·2 per cent. Owing to the cross reactivity of the $PGF_{2\alpha}$ antiserum with $PGF_{1\alpha}$, results are expressed as immunoreactive PGF. Standard curves, which were prepared using standards in assay buffer and in uterine washings, showed good parallelism for both compounds. The correlation coefficients for amount added:amount measured were 0·999 for PGF and 0·977 for PGE_2. The within- and between-assay coefficients of variation for PGF were 11 and 16 per cent and for PGE_2 they were 10 and 14 per cent, respectively. Sensitivity with reference to the standard curve (defined as the smallest concentration significantly different from the tube containing 0 pg) was 12·5 pg for PGF and 2·5 pg for PGE_2. For statistical analysis, concentrations which were below the limit of sensitivity were taken as equal to the limit of sensitivity.

Plasma PGFM radioimmunoassay

The method used to measure immunoreactive PGFM was as described by Watson et al (1987a). The main cross reactivities of the antibody were with 15-keto-$PGF_{2\alpha}$ 16 per cent, 13,14-dihydro $PGF_{2\alpha}$ 4 per cent, $PGF_{2\alpha}$ 0·4 per cent and 15-keto-13,14-dihydro PGE_2 1·7 per cent. Assay sensitivity with reference to the standard curve was 5 pg. Recovery of radiolabelled PGFM after extraction was 75 per cent. The within- and between-assay coefficients of variation were 10 and 22 per cent, respectively.

Statistical analysis

The effect of treatment on concentrations of prostaglandins in the lavage fluid was analysed by a one-way analysis of variance on log transformed data using horse as the blocking factor.

Results

Mean (± SEM) volume of washings recovered was 37·6 ± 0·6 ml. There was no growth of bacteria from any of the washings.

Before infusion of glycogen, prostaglandin concentrations in the fluid from all treatment groups were below the detection limits of the assays. Table 1 shows the mean concentrations of prostaglandins in lavage fluid at six and 24 hours after infusion of glycogen. In approximately 70 per cent of cases, higher concentrations of prostaglandins were present at six than at 24 hours after infusion of glycogen. As the periods of six and 24 hours were picked arbitrarily, and as individual mares appeared to reach peak prostaglandin concentrations at different intervals after infusion, statistical analysis was performed on the highest concentrations of prostaglandins measured at either six or 24 hours after infusion. Fig 1 shows the peak concentrations of PGF and PGE_2 measured in lavage fluid at either six or 24 hours after induction of endometritis. Significantly higher concentrations of uterine PGF ($P<0·01$) and PGE_2 ($P<0·05$) were measured in mares treated with progesterone, either alone or in combination with oestradiol, than in mares that had been treated with oestradiol or the oily vehicle. There were no significant differences in prostaglandin concentrations in uterine lavage fluid, however, between mares treated with progesterone alone or progesterone plus oestra-

TABLE 1: Concentrations of prostaglandins F (PGF) and E_2 (PGE_2) in uterine lavage fluid from steroid-treated ovariectomised mares after induction of acute uterine inflammation

		Concentration (pg ml^{-1}) in lavage fluid (mean ± SEM) Treatment			
	Time (hours) after infusion	Progesterone n = 4	Progesterone + oestradiol n = 4	Oestradiol n = 4	Vehicle n = 4
PGF	6	22,000 ± 7800	8525 ± 3702	825 ± 390	637 ± 322
	24	2600 ± 500	1720 ± 859	533 ± 84	625 ± 127
PGE_2	6	235 ± 49	346 ± 185	48 ± 15	80 ± 22
	24	133 ± 55	101 ± 47	35 ± 7	56 ± 12

diol, or between those treated with oestradiol alone or the oily vehicle.

The washing procedure by itself usually caused a small rise in PGFM concentrations in plasma which returned to pre-washing levels by 30 minutes (Fig 2). In the mare undergoing luteolysis, however, concentrations of plasma PGFM were still elevated at 30 minutes but had returned to pre-washing levels within six hours.

Discussion

Before intrauterine infusion of glycogen, concentrations of prostaglandins recovered in lavage fluid were below the limit of detection of the assays. This may have been due to the short period of hormone treatment, as elevated concentrations of PGF in uterine luminal fluid from ovariectomised mares have only been reported after 21 days of progesterone injections (Zavy et al 1984). However, in cycling mares, detectable concentrations of PGF were present at all stages of the oestrous cycle in uterine flushings collected by a more protracted lavage procedure than that used in the present study (Zavy et al 1978).

In the present study, treatment of mares with ovarian steroids had a large effect on the response of the uterus to an inflammatory stimulus. Mares treated with progesterone, either alone or in combination with oestradiol, produced much higher concentrations of prostaglandins in uterine fluid than mares treated solely with oestradiol or the oily vehicle. In cycling mares, uterine fluid from two mares in dioestrus also contained much higher concentrations of prostaglandins after intrauterine infusion of glycogen than fluid from one mare in oestrus (Watson et al 1987a).

Cervical dilation in the mare causes premature luteolysis (Hurtgen and Ganjam 1979). However, other workers, using a similar washing technique in successive cycles, found no alteration in cycle length (Strzemienski and Kenney 1984). Protracted vaginal and cervical manipulation and infusion of large volumes of saline (500 ml) can stimulate prostaglandin synthesis as measured by plasma concentrations of PGFM (Neely et al 1979, Betteridge et al 1985) and may explain the increased concentrations of PGF which were detected in lavage fluid throughout the oestrous cycle (Zavy et al 1978). The present study,

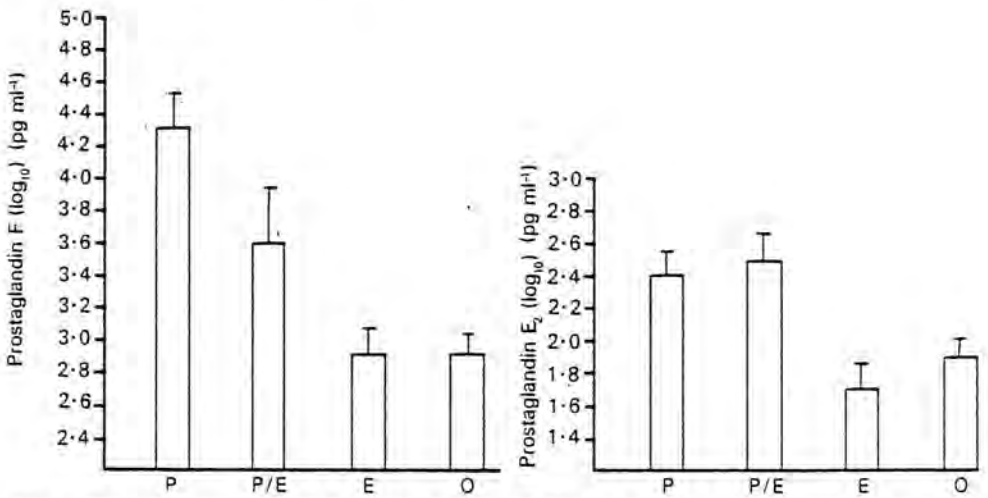

FIG 1: Peak concentrations (mean ± SEM) of PGF and PGE_2 in uterine washings collected from four ovariectomised mares at six or 24 hours after induction of endometritis. Mares were treated with progesterone (P), progesterone followed by oestradiol (P/E), oestradiol (E) or arachis oil (O)

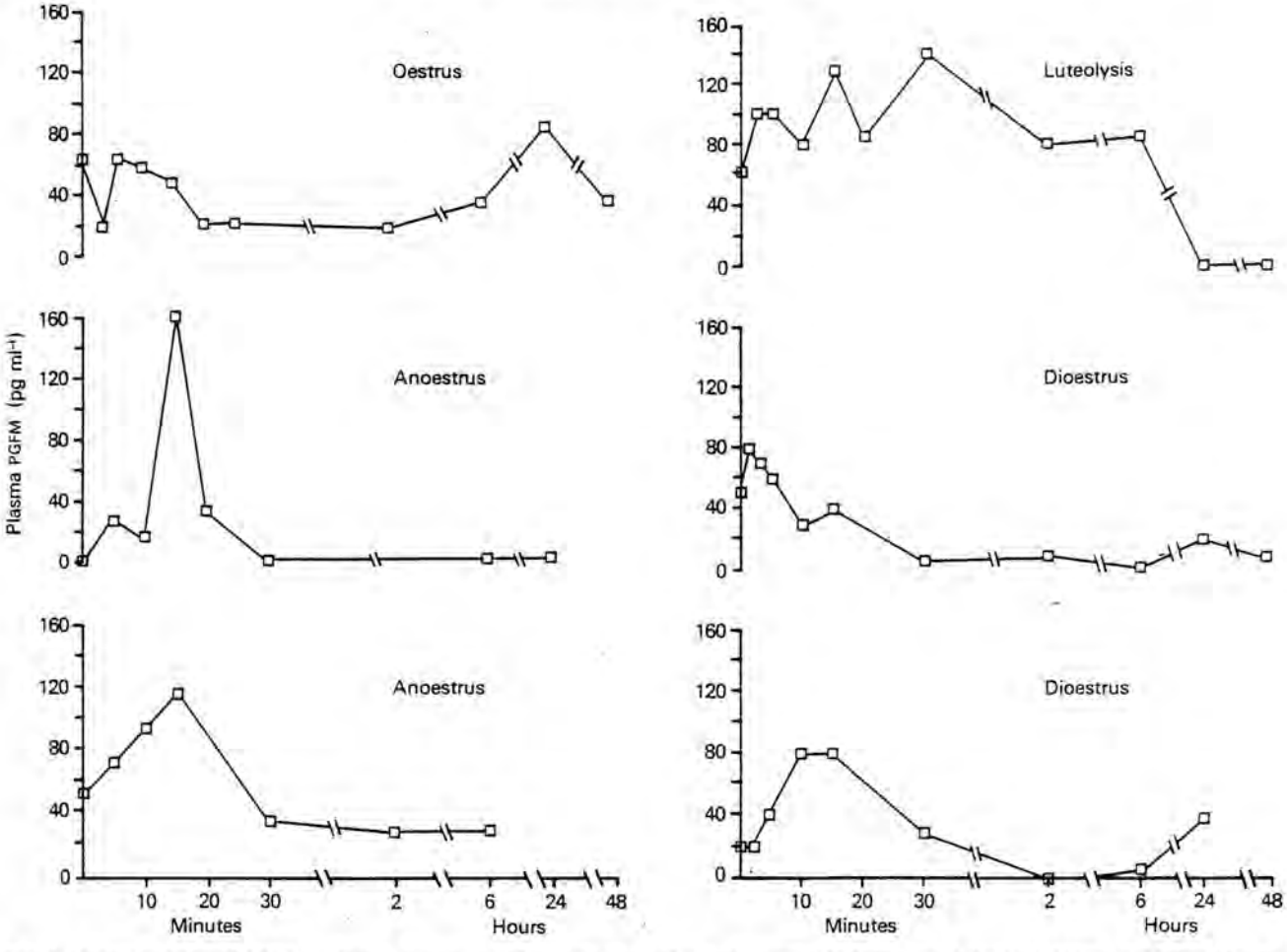

FIG 2: Concentrations of PGFM in plasma during and after collection of uterine washings from mares during seasonal anoestrus (n = 2), dioestrus (n = 2), oestrus (n = 1) or luteolysis (n = 1)

however, suggests that apart from late in dioestrus, lavaging procedures which employ small volumes of saline and minimal manipulation induce little sustained release of $PGF_{2\alpha}$ from the endometrium. It seems likely, therefore, that the major factor contributing to the prostaglandin concentrations in uterine washings was the induced inflammation with its associated influx of leucocytes and serum proteins into the uterus.

Many cells within the endometrium may contribute to prostaglandin synthesis, including white blood cells (Watson et al 1987a), connective and vascular tissues (Myatt et al 1975, Ritter et al 1983). The numbers of uterine luminal cells are not significantly different in the first six hours after the induction of endometritis in ovariectomised mares treated with progesterone, oestrogen or oily vehicle (Watson et al 1987b). In view of the large differences in prostaglandin concentrations between treatment groups, it is unlikely that cells or cell debris contributed significantly to an artefactual generation of prostaglandins in lavage fluid ex vivo. Therefore, it seems probable that, in addition to the leucocytes, the endometrium made a significant contribution to concentrations of prostaglandins measured in uterine washings collected after the infusion of mares which had been treated with progesterone alone or in combination with oestradiol.

Prostaglandins are not stored within tissues but are synthesised subsequent to a wide variety of stimuli. Substrate availability is regarded, therefore, as an important rate-limiting factor in prostaglandin synthesis (Ramwell et al 1977). In cattle, the endometrium contains increasing amounts of arachidonic acid, the principal substrate for prostaglandins, under the influence of high circulating concentrations of progesterone (Hansel et al 1975). In contrast it is thought that oestrogen triggers the increase in concentrations of the prostaglandin-synthetase enzyme, cyclo-oxygenase, which occurs at luteolysis in ewes (Huslig et al 1979). If similar events occur in mares, the relative deficiency of prostaglandin precursors within the endometrium of oestrogen-treated and

control mares may explain the lower concentrations of prostaglandins in washings collected from such mares.

The actions of elevated concentrations of PGE_2 and PGF in uterine secretions are possibly antagonistic. $PGE_{2\alpha}$ inhibits the proinflammatory effects of cells by elevating intracellular levels of cyclic AMP (Weissman et al 1980), whereas $PGF_{2\alpha}$ enhances the proinflammatory properties of cells (Zurier et al 1974). Similarly, antagonistic effects on the maintenance of the corpus luteum have been reported for these prostaglandins. PGE_2 apparently possesses antiluteolytic properties in sheep (Henderson et al 1977) and cattle (Gimenez and Henricks 1983), whereas $PGF_{2\alpha}$ is the naturally occurring luteolysin in many species, including the mare (Douglas and Ginther 1976). The precise interactions of these compounds within the uterus of the mare require investigation.

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Short Communications

nfluence of ovarian steroids on adherence *(in vitro)* of *Streptococcus zooepidemicus* to endometrial epithelial cells

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ntroduction

N mares, ewes and cattle it is well established that the uterus more susceptible to bacterial infection during periods of gh circulating concentrations of progesterone than when estrogen concentrations are elevated (Hawk, Turner and kes 1961; Hawk *et al* 1964; Ganjam *et al* 1984). Under the fluence of progesterone, deficiencies in cellular and humoral erine defence mechanisms have been reported (Blue, Brady, avidson and Kenney 1984; Asbury and Hansen 1987; atson, Stokes and Bourne 1987; Watson, Stokes, David and ourne 1987).

Bacteria attach firmly to mucosal surfaces in the oral cavity id intestine (Gibbons 1973). Furthermore, it appears that dherence is a prerequisite for successful colonisation of the enitourinary tract in women (Svanborg-Eden and Svenerholm 1978) and virulence of *Streptococcus pyogenes* is etermined by its ability to adhere to epithelial cells (Gibbons 73). Within the mare's uterus, the ability to adhere to ithelial cells may help pathogens to overcome the physical earance mechanisms described by Evans *et al* (1986).

Studies performed both *in vitro* and *in vivo* suggest that dherence of bacteria to epithelial cells of the genital tract of omen and rats is affected profoundly by ovarian steroids, but e specific effect varies with site, species and organism (Botta 79; Nishikawa and Baba 1985; Sobel and Kaye 1986). The esent study investigated whether these observations could extended to the mare and might provide some insight into e susceptibility of the progesterone-dominated uterus to erine infection.

aterials and methods

Four ovariectomised pony mares were injected daily for ven days with oestradiol benzoate (1 mg) or progesterone 00 mg) in a cross-over study (2 x 2). Fourteen days tervened between treatments and, after a further 14 days, e experiment was repeated. In oestradiol-treated mares ean (± sem) concentrations of plasma oestradiol-17β after ven days were 14.7 ± 2.7 pg/ml. Examination of endometrial opsies from mares on a similar hormone treatment regime owed tall columnar epithelium with cytoplasmic vacuolaon, margination of neutrophils within capillaries and straight es of glands, changes which are characteristic of oestrus in cycling mares. After one week of progesterone injections, mean (± sem) concentrations of plasma progesterone were 2.7 ± 0.6 ng/ml.

Histological examination of endometrial biopsies revealed low to medium columnar epithelium and increased gland density. These findings are similar to those of mares during dioestrus.

Uterine epithelial cells

These were obtained by scraping with an endometrial curette as described by Ricketts (1985). Cells were placed in sterile medium (Hanks Balanced Salt Solution buffered with Hepes, pH 7.3, containing 1 per cent heat inactivated newborn calf serum) and kept on ice. The cells were sedimented by centrifugation at 125 x *g* for 10 mins and the supernatant was discarded. The cell pellet was resuspended in medium and the cells were washed three times. Cell numbers were counted using a haemocytometer counting chamber and adjusted to 2 x 10^6/ml.

Bacteria

A strain of *Streptococcus zooepidemicus* was used which had been recovered from a mare clinically affected with endometritis. The bacteria were stored in aliquots at –70°C in Brain Heart Infusion Broth (BHIB) containing 10 per cent glycerol. Before assay the bacteria were grown up overnight in fresh BHIB, washed twice in sterile phosphate buffered saline (PBS; pH 7.3) and finally resuspended at 10^9/ml by calibration with a spectrophotometer (OD=0.9 at 640 mm).

Adherence assay

The bacterial suspension (0.5 ml) was added to cells (0.5 ml) in plastic stoppered tubes and incubated on ice for 60 mins. The tubes were then centrifuged at 125 x *g* for 5 mins and, after the fluid was discarded, the cells were washed three times in medium to remove non-adherent bacteria. After the final wash, cells were resuspended in 1.5 ml medium. An aliquot (0.1 ml) was added to 0.2 ml PBS and a smear of the cells was prepared using a cytocentrifuge (Dulin, Paape and Weinland 1982) and stained by Giemsa (Diff-Quick, American Hospital Supplies; Compton).

Slides were examined microscopically under oil immersion and the number of cells with more than ten adherent bacteria was counted for 100 cells.

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BLE 1: Bacterial adhesion to uterine epithelial cells from ariectomised mares receiving ovarian steroids

re	Per cent epithelial cells with more than 10 adherent bacteria	
	Oestradiol-treated	Progesterone-treated
	47	75
	19	39
	23	34
	29	46
sem	29.1 ± 7.22	46.3 ± 7.22

sults are the mean of duplicate experiments

tistical analysis

The means of the results for both experiments were culated and the effect of hormone treatment was analysed ng a paired *t* test.

ults

Microscopic examination of slides revealed *S zooepidemicus* nering to uterine epithelial cells to a varying degree. erine epithelial cells from ovariectomised mares treated h oestradiol demonstrated significantly ($P < 0.001$) lower cterial adherence (Table 1) than cells collected from mares ated with progesterone.

cussion

These results suggest that, in comparison with mares treated h progesterone, oestradiol treatment lowered bacterial nesion to uterine epithelial cells.

By performing the assay on washed cells *in vitro*, factors own to affect bacterial adhesion such as pH, mucin rsons, Greenspan, Moore and Mulholland 1977) and munoglobulins (Svanborg-Eden and Svennerholm 1978) re excluded. Thus the observed changes in cell receptiveness adherence by *S zooepidemicus* were apparently due to dification of properties of the cell surface. It is known that morphology of endometrial epithelial cells in mares is ected profoundly by ovarian steroids (Kenney 1978) and it y be that cellular receptors for streptococci in mares are ntrolled by concentrations of oestradiol and/or proges-one.

Studies performed on women and rats have shown that stradiol treatment increased adherence of *Escherichia coli* to ginal and urinary tract epithelial cells (Botta 1979; Reid, ooks and Bacon 1983; Sobel and Kaye 1986), but decreased nerence to uterine epithelial cells of rats (Nishikawa 1985; shikawa and Baba 1985). *Streptococcus zooepidemicus* is ognised to be an opportunistic pathogen and requires mary predisposing factors before it can become established an infective agent (Bryans and Moore 1972). Thus the reased bacterial adherence to cells from a progesterone-minated uterus may be important in increasing uterine ceptibility to infection.

It would be interesting to extend this study to investigate erine epithelial cells from mares susceptible to persistent dometritis, particularly in view of the association between ceptibility to recurrent urinary tract infections in women d increased bacterial adherence to epithelial cells *in vitro* wler and Stamey 1977; Svanborg-Eden and Svennerholm 8).

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Plasma cell numbers in uteri of mares with persistent endometritis and in ovariectomised mares treated with ovarian steroids

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mmary

mmunoglobulins A, G and M were localised by mmunoperoxidase staining of endometrial sections from ariectomised mares. Treatment with progesterone or stradiol-17β did not significantly affect numbers of cells creting any of the isotypes. Mares with persistent dometritis did not have significantly greater numbers of dometrial plasma cells than genitally-normal mares.

troduction

HERE is substantial evidence for local production of antibody the equine endometrium. The presence of plasma cells within e equine endometrium has been demonstrated by both munofluorescence (Kenney and Khaleel 1975; Mitchell *et al* 82) and immunoperoxidase (Widders, Stokes, David and urne 1985a; Waelchli and Winder 1987) staining techniques. rther, proportionately higher concentrations of munoglobulins have been measured in uterine secretions than serum (Mitchell *et al* 1982; Widders, Stokes, David and urne 1984) and endometrial explants cultured *in vitro* thesise significant amounts of immunoglobulin (Ig)G, IgA and M (Watson 1987), thus confirming the potential of the roductive tract for local production of immunoglobulin.

Mares with persistent endometritis have significantly higher ncentrations of immunoglobulins in uterine secretions than nitally-normal mares (Asbury, Halliwell, Foster and Longino 80; Williamson, Dunning, O'Conner and Penhale 1983). In nitally-normal mares, measurement of immunoglobulin ncentrations in uterine secretions at different stages of the strous cycle has produced conflicting results. Higher ncentrations of IgG have been measured in dioestrus, with no nges in IgM or in IgA between phases (Mitchell *et al* 1982). In er studies, however, either uterine IgA (Asbury *et al* 1980) or h IgA and IgG (Widders, Stokes, David and Bourne 1985b) e been reported to be higher during oestrus while the trend in M concentrations was the reverse (Widders *et al* 1985b). The nbers of endometrial plasma cells have not been found to lect these cyclical fluctuations in immunoglobulin ncentrations in uterine secretions (Widders *et al* 1985a; elchli and Winder 1987). As plasma cell numbers have only been studied in cycling mares, it is difficult to attribute changes in numbers to either oestrogen or progesterone, because wide fluctuations in concentrations of these hormones normally occur during the oestrous cycle.

The present study investigated the direct effect of ovarian steroids on plasma cell numbers in endometria of ovariectomised mares treated with physiological amounts of oestradiol and progesterone.

Materials and methods

Four ovariectomised pony mares with no histological evidence of endometrial inflammatory changes were treated daily for seven days with intramuscular injections (4 ml) of oestradiol benzoate (1 mg), progesterone (100 mg) or the oily vehicle, arachis oil (Intervet Laboratories; Cambridge) using a blocked experimental design so that each mare received all three treatments. This treatment regime had been shown to produce changes in the endometria and in plasma hormone concentrations consistent with oestrus, dioestrus and anoestrus, respectively (E. D. Watson, unpublished data). On Day 7, endometrial biopsies were collected. A period of two weeks elapsed between treatments.

Endometrial biopsies were collected from a further group of 10 genitally-normal mares and 16 mares which had persistent endometritis as defined previously (Watson *et al* 1987). These mares were at various stages of the oestrous cycle when sampled.

The biopsies were fixed in Carnoy's solution for 15 to 24 h. Subsequent preparation of tissues for microscopy and staining were performed as described by Widders *et al* (1985a). Primary antisera were sheep anti-horse IgA, mouse anti-horse IgG_{ab} (both raised in this laboratory) and rabbit anti-horse IgM (Nordic Laboratories; Maidenhead). All were shown by immunodiffusion to be specific for each isotype. Optimal working dilutions were prepared for each antiserum. Secondary antisera were peroxidase conjugated pig anti-sheep IgG and sheep anti-rabbit IgG (prepared in this laboratory) and sheep anti-mouse IgG (Sigma Chemical; Poole). All conjugated antisera were used at a dilution of 1:50.

Numbers of plasma cells secreting each of of the isotypes were scored by counting stained cells in 100 high power fields (400 x). Where the amount of mucosa available from the biopsy was limited, at least 50 fields were counted. Cell counts were analysed using an analysis of variance on $\log_{10}$ transformed data. The effects of hormone were examined for each isotype with horse as the blocking factor. The differences between genitally-normal mares and mares with endometritis were analysed by a students *t*

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ABLE 1: Mean (±sem) number of plasma cells (log_{10}) per 100 gh power fields in the endometrium of four mares

	Treatment		
	Oestradiol	Progesterone	Arachis oil
G	1.9 ± 1.4	2.1 ± 1.7	1.9 ± 1.8
A	1.3 ± 0.7	1.6 ± 0.9	1.5 ± 1.1
M	1.8 ± 1.2	2.0 ± 1.4	1.8 ± 1.4

t on log-transformed data. Values of $P<0.05$ were considered nificant.

esults

Specificity of the immunoperoxidase staining technique was sured by the established specificity of the antisera. The hnique was validated by demonstration of absence of staining ien either antiserum or conjugate was omitted.

Plasma cells staining for IgG were most numerous, followed by M and IgA (Table 1). Hormone treatment did not significantly luence occurrence of any of the isotypes.

Total numbers (log_{10}) of endometrial plasma cells were not nificantly different between mares with endometritis (2.9 ± 3) and genitally-normal mares (1.9 ± 1.43).

scussion

The results of the present study, in which ovarian steroids were ministered to ovariectomised mares, are similar to results tained in cycling mares (Widders *et al* 1985a). In both studies, sma cells secreting IgG were the most numerous, but there was significant pattern in plasma cell numbers for any of the otypes which could be attributed to the effect of ovarian roids.

Ovarian steroids have been found to influence variation in dometrial plasma cell numbers in a number of species. aximum numbers have been reported in tissue from the sow ussein, Newby and Bourne 1983), rat (Wira *et al* 1980) and ouse (McDermott, Clark and Bienenstock 1980) under the luence of oestrogen. In human endometrium, however, plasma l numbers peaked shortly after ovulation (Tourville, Orga, pes and Romansi 1970).

The results of the present study do not correspond to reports of vated IgG and IgM in equine uterine secretions collected ring dioestrus (Mitchell *et al* 1982; Widders *et al* 1985b) but it likely that mechanisms other than synthesis affect ncentrations of uterine luminal immunoglobulins, such as the fects of ovarian hormones on blood flow and vascular meability in the endometrium.

In mares with persistent endometritis, greatly increased mbers of endometrial plasma cells were sometimes found mpared with genitally-normal mares. The very high number of sma cells in the endometria of those mares with endometritis s presumably a direct result of uterine infection and these cells may contribute to the elevated concentrations of immunoglobulins measured in uterine secretions (Asbury *et al* 1980; Williamson *et al* 1983). However, in these mares a large proportion of the immunoglobulins within the uterine lumen will originate from the serum leakage associated with inflammation, rather than from synthesis by plasma cells.

In conclusion, treatment of ovariectomised mares with ovarian steroids did not affect numbers of plasma cells secreting IgG, IgA and IgM within the endometrium. Total plasma cell numbers were not significantly different between genitally-normal mares and mares with persistent endometritis.

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Influence of arachidonic acid metabolites in vitro and in uterine washings on migration of equine neutrophils under agarose

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The influence of arachidonic acid metabolites on migration of equine neutrophils under agarose was investigated. Leukotriene B_4 (LTB_4) was chemotactic at concentrations between 0·1 and 1000 ng ml^{-1} and prostaglandin E_2 (PGE_2) at 1 and 10 ng ml^{-1} but not at higher or lower concentrations. Prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) was not chemotactic for equine neutrophils at any concentration. Random migration was significantly inhibited ($P<0·05$) by suspension of neutrophils in LTB_4 (0·1 to 1000 ng ml^{-1}). Migration was also inhibited ($P<0·05$) by suspension in low concentrations of PGE_2 (0·1 and 1 ng ml^{-1}) and $PGF_{2\alpha}$ (0·1 ng ml^{-1}) but not at high concentrations. There was a significant positive correlation between random migration of neutrophils suspended in uterine washings from persistently endometritic mares and concentrations of endogenous PGF ($P<0·002$) and PGE_2 ($P<0·05$) in washings. Thus certain metabolites of arachidonic acid affect migration of equine neutrophils and may play a significant role in recruitment of neutrophils to sites of inflammation in the horse.

THE earliest response to acute injury is usually characterised by vasodilation and increased permeability of local blood vessels followed by influx of neutrophils (Weissmann 1980). Metabolites of arachidonic acid, the prostaglandins and the leukotrienes, are important mediators at sites of inflammation (Weissmann 1980, Higgs 1982). Individual prostaglandins and leukotrienes have been shown to act directly as mediators of inflammation and also to potentiate the effect of other inflammatory mediators such as histamine, bradykinin and complement C_{5a} (Eakins et al 1980, Weissmann 1980, Higgs et al 1981). Owing to their presence at sites of inflammation, the effect of arachidonic acid metabolites on neutrophil migration has attracted considerable attention. Although several of the leukotrienes stimulate chemokinesis and chemotaxis of neutrophils from humans, rats and rabbits the most potent is leukotriene B_4 (LTB_4) (Ford-Hutchinson et al 1980, Palmer et al 1980). The influence of prostaglandins on neutrophil migration has yet to be fully defined. However, two prostaglandins, prostaglandin $F_{2\alpha}$ ($PGF_{2\alpha}$) and prostaglandin E_2 (PGE_2) have been shown to have no apparent effect on chemotaxis of rabbit, rat or human neutrophils but, respectively, to increase and to inhibit chemokinesis of human neutrophils (Diaz-Perez et al 1976, Goetzl and Gorman 1978).

Both immunoreactive LTB_4 and PGE_2 have been detected in experimentally induced inflammatory exudate from horses (Higgins and Lees 1984, Higgins et al 1986). Concentrations of PGE_2 and PGF in uterine washings increased after uterine inflammation was induced in genitally normal mares compared with concentrations measured before infusion of the irritant (Watson et al 1986). High concentrations of prostaglandins were also measured in washings from mares clinically affected with persistent endometritis (Watson et al 1987). The present study was designed to investigate the influence of LTB_4, $PGF_{2\alpha}$ and PGE_2 on migration of equine neutrophils under agarose at concentrations which might be present in acute inflammatory exudate. In addition, the effect on neutrophil locomotion of suspending equine neutrophils in uterine washings was investigated.

Materials and methods

Venous blood was collected from two seasonally anoestrous mares, one ovariectomised mare and one gelding for studies with LTB_4. In studies using prostaglandins, blood was collected from one gelding. In control plates, the cells from the gelding migrated the average distance of those from the three mares.

Leucocytes were isolated from blood by the method of Zinkl and Brown (1982). Leucocytes were counted using a haemocytometer and viability was assessed by exclusion of trypan blue dye. After cell differentiation using a cytospin preparation stained with Giemsa, the neutrophil concentration was adjusted to 10^7 ml^{-1} in Hanks' balanced salt solution buffered with Hepes at pH 7·3 (HBSS).

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Neutrophil migration assays

The locomotion of neutrophils under agarose was studied by a modification of the method of Zinkl and Brown (1982). Solutions of agarose in distilled water were heated to boiling at concentrations of 1·5 per cent for the study of directional migration towards a chemoattractant and 2·3 per cent for the study of random migration. The hot agarose solutions were mixed with an equal volume of medium containing 0·4 per cent bovine serum albumin in HBSS for directional migration plates and 20 per cent heat treated newborn calf serum (Gibco) in HBSS for random migration plates. The solutions were kept at 56°C until they were dispensed in 5·5 ml volumes into 10 × 35 mm (depth × diameter) tissue culture dishes (Sterilin). The agarose plates were allowed to gel at room temperature and then were refrigerated to increase gel firmness. A template was used to punch four sets of three wells in each plate of agarose. The wells had a diameter of 2·4 mm and in the directional migration plates each set of wells was in a straight line with edges 2·4 mm apart. In the random migration plates a set of three wells were placed in a triangular pattern in each quarter of the plate.

In directional migration plates the chemoattractant (30 μl) was added to the middle well of each set of three and cells (30 μl) were added to the outer wells. Each set of three wells contained cells from one horse. Four replicate plates were used.

In plates used for random migration, 30 μl of cells from each horse were added to one of the sets of three wells on duplicate plates.

The plates were incubated in a humidified incubator at 37°C for two and a half hours. After removal from the incubator the cells were fixed with absolute methanol for 30 minutes, followed by removal of the gel. The plates were stained with Leishman's and then air dried.

On chemotaxis plates there was no random migration from the side of the well furthest from the chemoattractant. Movement of cells was evaluated by the leading front method using the 10× objective of the microscope fitted with a 10× ocular which contained a grid whose distances had been calculated using a stage micrometer. Distance moved was measured from the edge of the well to the last line of 10 squares which contained at least one neutrophil in five separate squares. On plates used for random migration, a reading was taken from each of four sides of every well.

Chemotactic factors

In control plates for chemotaxis, fresh horse serum from a gelding was used as chemoattractant. On the day of assay, stock solutions of LTB_4 (Miles), PGE_2 (Sigma) and $PGF_{2\alpha}$ (Sigma) were prepared in ethanol (1 mg ml^{-1}). The required volumes were dried down in siliconised glass tubes at 45°C under a stream of nitrogen and fresh serum from a gelding was added to obtain the required concentrations (0·1, 1, 10, 100, 1000 ng ml^{-1}). The solutions were stored at 4°C until use. For random migration plates, the test substances were prepared in HBSS instead of serum and were used as the suspension medium for the neutrophils.

Uterine washings

Uterine washings were collected from 10 mares with normal genital tracts and from 10 mares with persistent endometritis using 80 ml sterile phosphate buffered saline (pH 7·0). Although all of these mares were cycling at the time of collection none was at luteolysis (as determined by oestrous dates and plasma progesterone and oestrogen results). Mares with persistent endometritis had not previously received antibiotic treatment within a month of sampling. The collection and treatment of samples was as described by Watson et al (1987). Cultures of washings on blood agar plates at 37°C for 48 hours showed that none of the washings from mares with persistent endometritis was infected with bacteria at the time of sampling. Neutrophils from the gelding described previously were suspended in washings at a concentration of 10^7 ml^{-1} and the effect on neutrophil locomotion was studied on chemokinesis plates.

Total protein assay

Total protein in uterine washings was measured using a microprotein rapid stat diagnostic kit (Sherwood Medicals).

PGF and PGE_2 radioimmunoassays. Concentrations of PGF and PGE_2 were measured directly in uterine washings by the method of Watson et al (1987). Antisera were raised in rabbits against BSA-conjugates for both hormones. Anti-PGF was supplied by Dr H. Kindahl, Uppsala, and anti-PGE_2 by the Sigma Chemical Co. The main cross reactivities (over 0·5 per cent) of the anti-PGF were $PGF_{2\alpha}$ 100 per cent, $PGF_{1\alpha}$ 75 per cent and $PGF_{2\beta}$ 1·5 per cent and of the anti-PGE_2 was PGE_1 3·2 per cent. Approximately 30 per cent of the radioactive prostaglandin was bound to antibody at a final dilution of 1:300 (PGF) and 1:30 (PGE_2) in the absence of unlabelled hormone. Standard curves prepared using samples in assay buffer and uterine washings showed good parallelism. The coefficient of correlation for amount added: amount measured was 0·999 for PGF and 0·977 for PGE_2. The within-assay coefficient of variation for PGF was 11 per cent over the range 50 to 500 pg and for PGE_2 was 10 per cent over the range 12·5 to 50 pg. Sensitivity with respect to the standard curve was 12·5

pg for PGF and 1·25 pg for PGE_2. All samples were included in one assay.

Statistical analysis

Results were analysed using a *t* test and Pearson's correlation test and differences were considered significant when $P<0·05$. All means are quoted ± SEM.

Results

Neutrophil chemotaxis was significantly greater towards serum containing LTB_4 than towards control serum and distance travelled was dose-related between 1 and 100 ng LTB_4 ml^{-1} but not at lower concentrations (Fig 1). Chemotaxis was also significantly increased towards serum containing 1 and 10 ng PGE_2 ml^{-1} but not at lower or higher concentrations. Serum containing $PGF_{2\alpha}$ did not increase chemotaxis at any concentration tested.

When neutrophils were suspended in LTB_4 migration was significantly reduced and the inhibition increased with increasing concentrations of LTB_4 (Fig 2). Migration was also significantly reduced with 0·1 ng ml^{-1} of PGE_2 and $PGF_{2\alpha}$ and with 1 ng PGE_2 ml^{-1} but addition of higher concentrations had no effect.

Migration was not significantly different between neutrophils suspended in washings from mares with persistent endometritis (1·03 ± 0·06 mm) and normal mares (1·09 ± 0·05 mm). However, migration was less than that of cells suspended in phosphate buffered saline ($\bar{x}$ = 1·22 mm). Distance migrated was not related to protein concentration of washings ($r = 0·098$). In genitally normal mares there was no relationship between migration and concentrations of PGF (0·6 ± 0·2 ng ml^{-1}) and PGE_2 (39·4 ± 18·6 pg ml^{-1}) in washings. However, in mares with persistent endometritis, there was a significant direct correlation between migration and concentrations of PGF (2·6 ± 0·8 ng ml^{-1}; $r = 0·82$; $P<0·002$) and PGE_2 (113·5 ± 43·7 pg ml^{-1}; $r = 0·65$; $P<0·05$) in washings.

Discussion

Leukotriene B_4 was a potent chemotaxin for equine neutrophils. Maximal activity was obtained at concentrations of 100 and 1000 ng LTB_4 ml^{-1} serum which is a higher concentration than that reported for other species (Palmer et al 1980, Craven 1986). Significant migration was observed at concentrations as low as 0·1 ng ml^{-1} which is similar to human neutrophils (Palmer et al 1980, Craven 1986). However bovine neutrophils were not sensitive to such low concentrations (Craven 1986). In the present study, the migration observed on random migration plates did not only reflect chemokinesis as there was a concentration gradient which decreased as the neutrophils migrated from the well. Thus, in contrast to results from other species in which chemokinesis was consistently enhanced by LTB_4 (Ford-Hutchinson et al 1980, Palmer et al 1980), chemokinesis of equine neutrophils was reduced as concentrations of LTB_4 increased. The potent chemotactic action of LTB_4 appeared to overwhelm its chemokinetic activity and neutrophils were strongly inhibited from leaving the wells at concentrations of 100 and 1000 ng ml^{-1}, possibly demonstrating immobilisation (desensitisation) by saturing levels of chemotaxin.

In other species high concentrations of $PGF_{2\alpha}$ and PGE_2 (1 to 25 μg ml^{-1}) were not chemotactic (Diaz-Perez et al 1976, Goetzl and Gorman 1978). However,

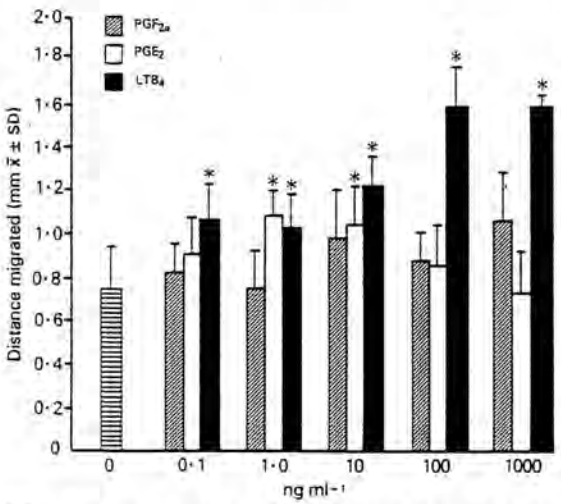

FIG 1: Chemotaxis of equine neutrophils towards serum containing $PGF_{2\alpha}$, PGE_2 and LTB_4. *Migration significantly greater than towards control wells containing serum only ($P<0·05$)

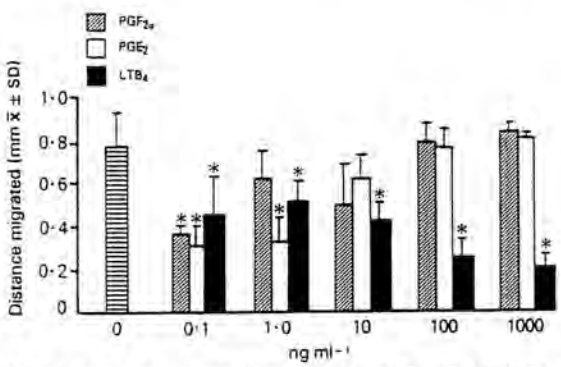

FIG 2: Migration of neutrophils suspended in $PGF_{2\alpha}$, PGE_2 and LTB_4 on chemokinesis plates. *Migration significantly less than from control wells containing neutrophils suspended in HBSS ($P<0·05$)

in the present study, PGE_2 in serum exerted chemotactic activity for equine neutrophils at concentrations of 1 and 10 ng ml^{-1} which were considerably lower concentrations than those used by other workers. Similarly, effects of prostaglandins on chemokinesis have been observed by suspending cells in high concentrations of PGE_2 and $PGF_{2\alpha}$ (Goetzl and Gorman 1978). Equine neutrophils suspended in the low concentrations of PGE_2 and $PGF_{2\alpha}$ used in the present study showed inhibition of migration from the well probably due to a chemotactic effect at 0·1 ng ml^{-1} and also at 1 ng ml^{-1} in the case of PGE_2. The chemotactic activity demonstrated by PGE_2 was not expected as PGE_2 tends to increase the intracellular levels of cyclic AMP. When levels of cyclic AMP are raised in inflammatory cells their major effect is to inhibit parameters of cellular function such as chemotaxis (Weissmann et al 1980).

It is interesting to note that the chemotactic activity of PGE_2 at 1 and 10 ng ml^{-1} was not significantly less than that of LTB_4 which contrasts with the highly potent chemotactic effect of LTB_4 recorded in other species (Ford-Hutchinson et al 1980, Palmer et al 1980). However, the range of concentrations over which LTB_4 was chemotactic was very much wider than that of PGE_2. In the present study the test substances were suspended in serum unlike other studies in which buffer was used. It has been suggested that serum is chemotactic for neutrophils migrating under agarose due to activation of complement by the agarose (Gray et al 1982). Thus it may be that the prostaglandins and leukotriene modified this interaction and that their direct chemotactic effect was not being tested in the present study. During collection and clotting of blood, synthesis of prostaglandins by platelets has increased endogenous concentrations of $PGF_{2\alpha}$ and PGE_2 from between 9 and 131 pg ml^{-1} to between 1·5 and 7 ng ml^{-1} (Morris et al 1981). However, this order of increase in prostaglandin concentrations would not substantially alter final concentrations of prostaglandins in the serum used as the chemotaxin. Concentrations of thromboxane in human serum increased to as high as 308 ng ml^{-1} during clotting (Morris et al 1981). However, in the horse, the mean concentrations measured in serum from 18 horses was only 29 ng ml^{-1} (Hardee et al 1986). Studies using human neutrophils have reported chemotactic activity for thromboxane B_2 (Boot et al 1976) but other work has suggested that thromboxane has little effect on neutrophil migration (Goetzl and Gorman 1978). By comparing migration towards serum containing the test prostaglandin or leukotriene with control serum, the possible effects of thromboxane on neutrophil locomotion could be ignored.

Concentrations of LTB_4 of 1·7 to 3·2 ng ml^{-1} have been measured by radioimmunoassay in inflammatory exudate induced by a tissue cage model of inflammation in horses (Higgins and Lees 1984). Using the same model, mean (± SEM) concentrations of PGE_2 of 66·4 ± 19·1 ng ml^{-1} have been recorded (Higgins et al 1986). In 16 mares with persistent endometritis, none of which was at luteolysis, total concentrations of PGE_2 (ng ml^{-1} × volume recovered) measured in uterine washings by radioimmunoassay ranged from below the limit of sensitivity of the assay to 27 ng ($\bar{x}$ = 6·5 ng). Also using radioimmunoassay, concentrations of PGF in the same mares ranged from below the limit of sensitivity of the assay to 480 ng ($\bar{x}$ = 142 ng) (Watson et al 1987). It has been calculated that the mean volume of native secretion within the mare's uterus is approximately 6·5 ml (Widders et al 1984). Mean recovery of washings was 75 per cent. Thus the mean concentration of PGE_2 in undiluted secretions was approximately 1·3 ng ml^{-1} and that of PGF was 29·0 ng ml^{-1}. Thus it would appear that sufficient concentrations of prostaglandins and LTB_4 may be present at sites of inflammation in horses to exert a significant effect on migration and locomotion of neutrophils.

Despite the high dilution factor which inevitably results from collection of uterine secretions using large volumes of phosphate buffered saline, sufficient chemotaxins were present in washings from endometritic mares to affect neutrophil migration. Endogenous PGF and PGE_2 in washings affected migration in a manner similar to results obtained by in vitro addition of prostaglandins to neutrophils in HBSS. However, other factors, such as LTB_4 and activated complement were probably present in the washings and may also have influenced neutrophil migration. It is interesting that migration was not related to protein concentrations in washings as many chemotaxins are proteins or protein fragments. This implies that the neutrophil migration was controlled by the production of specific factors in uterine secretions in response to inflammation. In genitally normal mares, very low concentrations of prostaglandins were present in washings and these were not correlated with migration.

In conclusion, LTB_4, PGE_2 and $PGF_{2\alpha}$ exerted significant effects on chemotaxis and, or, chemokinesis of equine neutrophils. It is likely that LTB_4 and PGE_2 play a significant role in the horse in recruitment of neutrophils to sites of inflammation. These observations support the hypothesis that in the horse, as in other species, the use of dual inhibitors of cycloxygenase and lipoxygenase such as the experimental compound BW 755C (Higgs 1983) could represent a significant improvement on existing non-steroidal anti-inflammatory drugs.

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Concentrations of immunoreactive leukotriene B_4 in uterine lavage fluid from mares with experimentally induced and naturally occurring endometritis

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Watson, E.D., Stokes, C.R. & Bourne, F.J. Concentrations of immunoreactive leukotriene B_4 in uterine lavage fluid from mares with experimentally induced and naturally occurring endometritis. *J. vet. Pharmacol. Therap.* **11**, 130–134.

Acute endometritis was induced in ovariectomized pony mares by infusion of a 1% solution of oyster glycogen. Maximum concentrations of immunoreactive leukotriene B_4 in uterine washings coincided with the greatest rate of infiltration of neutrophils into the uterine lumen. Concentrations of immunoreactive leukotriene B_4 decreased to basal levels 6 h after infusion and were unaffected by administration of ovarian steroids to ovariectomized mares. Uterine washings from mares with persistent endometritis did not contain significantly different concentrations of leukotriene B_4 from genitally normal mares.

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INTRODUCTION

Persistent endometritis is a major cause of subfertility in thoroughbred mares (Peterson *et al.*, 1969; Hughes & Loy, 1975). These mares are prone to persistent bacterial infection of the uterus whenever challenged. It is important to understand the pathogenesis of the inflammatory process in attempting to formulate rational therapy for mares with persistent endometritis.

Arachidonate metabolites have been implicated in other species as important inflammatory mediators. Several arachidonate metabolites, including leukotriene B_4 (LTB_4), have been identified in experimentally induced inflammatory lesions in horses (Higgins *et al.*, 1986). Leukotriene B_4 may mediate or enhance leucocyte recruitment during inflammation due to its potent chemotactic properties for neutrophils of the horse (Watson *et al.*, 1987a) and other species (Ford-Hutchinson *et al.*, 1980; Palmer *et al.*, 1980). Leukotriene B_4 also enhances plasma leakage when present in combination with PGE_2 (Higgs *et al.*, 1981).

The present study investigated both the concentrations of LTB_4 in uterine secretions of mares with persistent endometritis and the appearance of LTB_4 in uterine secretions after experimental induction of endometritis. In addition, the influence of ovarian steroids on uterine luminal LTB_4 concentrations was determined.

MATERIALS AND METHODS

Induction of endometritis

Four ovariectomized pony mares with no

evidence of endometritis on histological examination of biopsies, were treated daily for 8 days sequentially with 1 mg oestradiol benzoate, 100 mg progesterone or the oily vehicle arachis oil (Intervet Laboratories, Cambridge) in a cross-over experimental design so that each mare received all treatments. The period between treatments was 14 days. This dosage regimen has been shown to produce steroid concentrations similar to those measured during oestrus, dioestrus and anoestrus, respectively (Watson, 1986). On day 7 of treatment a uterine wash was performed using 40 ml sterile phosphate buffered saline (PBS; pH 7.0) introduced via a Foley catheter (24 FG, 30 ml cuff). Immediately after the washing procedure a solution of 1% oyster glycogen (Sigma Chemical Co., Poole) prepared in PBS (50 ml) was infused. Uterine washings were collected after 1, 3, 6 and 24 h. It has been shown that serial flushing of the uterus at intervals such as these does not markedly alter the inflammatory response to infusion of an irritant (Williamson *et al.*, 1987).

Clinically affected mares

Uterine washings were collected from mares with persistent endometritis ($n = 14$) and from genitally normal mares ($n = 14$). Washings were assayed for concentrations of immunoreactive LTB_4.

Treatment of washings

Washings were kept on ice until centrifuged at 125 *g* for 10 min at 4°C. The cell pellet was resuspended in PBS and leucocytes counted using a haemocytometer. Viability was assessed by exclusion of trypan blue dye. A differential leucocyte count was performed on a cytospin preparation. The supernatant was further centrifuged at 10,000 *g* for 30 min, aspirated and stored in aliquots at −70°C.

Radioimmunoassay of LTB_4

Concentrations of immunoreactive LTB_4 were measured in unextracted samples using a specific leukotriene B_4 [^{3}H] Kit (Amersham U.K. Ltd, High Wycombe). The assay has been described by Salmon *et al.* (1981). A range of concentrations of LTB_4 were added to a uterine washing and eight replicates were assayed at each concentration. This curve gave good parallelism with a standard curve prepared in assay buffer. The sensitivity of the assay was < 3 pg/tube. Both within- and between-assay coefficients of variation were 5%.

Statistical analysis

The effect of ovarian steroids on concentrations of LTB_4 in uterine washings was determined by analysis of variance. Differences between mares with persistent endometritis and genitally normal mares were analysed by a *t*-test. Values of $P < 0.05$ were considered significant.

RESULTS

Three hours after induction of endometritis, there was a high degree of neutrophil infiltration into the uterine lumen which was maintained for at least 24 h (Fig. 1). Concentrations of LTB_4 in uterine secretions peaked between 1 and 3 h; thereafter concentrations fell markedly and remained low. Treatment of ovariectomized mares with ovarian steroids did not significantly alter LTB_4 concentrations compared with control mares (Table I).

Uterine washings from mares clinically affected with persistent endometritis did not contain significantly different concentrations of LTB_4 (73.2 ± 29.6 pg/ml) from genitally normal mares (49.3 ± 8.2 pg/ml).

DISCUSSION

Maximum concentrations of LTB_4 coincided temporally with the maximum rate of infiltration of neutrophils. Peak concentrations were reached earlier than those described in subcutaneous lesions in rats (Simmons *et al.*, 1983) and horses (Higgins & Lees, 1984) probably reflecting the more rapid influx of

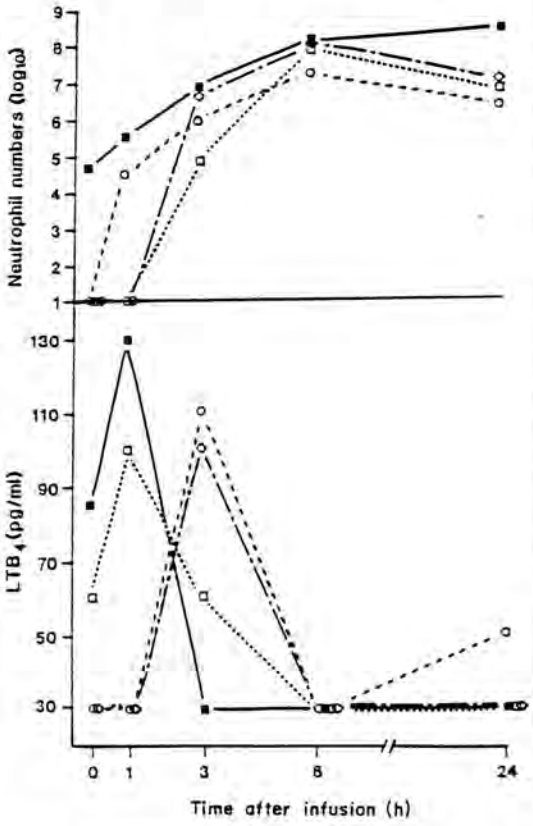

FIG. 1. Concentrations of LTB_4 and total numbers of neutrophils in uterine washings after induction of endometritis by infusion of oyster glycogen to four ovariectomized mares. Symbols represent individual mares.

neutrophils into the highly vascularized uterus than into subcutaneous sites.

Maximum concentrations of LTB_4 in washings were considerably lower than PGF and PGE_2 (Watson *et al.*, 1987b), occurred earlier and declined faster than PGF and PGE_2 which reached high concentrations at 6 and 24 h (Watson *et al.*, 1987b; Watson *et al.*, 1987c). Other workers have suggested that prostaglandins and LTB_4 may be derived from different sources at inflammatory sites (Simmons *et al.*, 1983; Higgins *et al.*, 1986) which may account for the observed differences. Furthermore, neutrophils do not metabolise PGE_2 (Salmon *et al.*, 1982) whereas leukotriene B_4 is readily metabolised by both human (Salmon *et al.*, 1982) and rat (Simmons *et al.*, 1983) neutrophils. This would further assist in the persistence of prostaglandins at inflammatory sites after LTB_4 has fallen to low concentrations. It is possible that a proportion of the LTB_4 was generated after collection of uterine washings from continued synthesis by leucocytes *ex vivo*. In order to minimize this artifactual generation, samples were kept on ice and centrifuged as soon as possible after collection.

The concentrations of LTB_4 measured in uterine secretions would be sufficient (total = 1.2–17.6 ng) to have a chemotactic effect on equine neutrophils (Watson *et al.*, 1987a). Thus, in the horse it is likely that LTB_4 may participate in mediating cell recruitment to

TABLE I. Concentrations of LTB_4 in uterine washings after induction of endometritis by infusion of oyster glycogen to ovariectomized mares treated with ovarian steroids

Time after Infusion (h)	Concentration ($\bar{X}$ ± SEM) of LTB_4 in uterine washings (pg/ml)		
	Oestrogen-treated $n = 4$	Progesterone-treated $n = 4$	Controls $n = 4$
0	41.0 ± 7.5	52.5 ± 16.5	51.3 ± 13.3
1	46.3 ± 10.9	55.3 ± 10.1	72.5 ± 29.2
3	92.5 ± 21.0	40.3 ± 7.0	75.0 ± 18.5
6	144.5 ± 75.5	< 30	< 30
24	132.5 ± 152.5	< 30	35.0 ± 5.0

the inflamed uterus, or at least modulate the actions of other chemotactic agents. From the results of the present study it was not possible to distinguish whether the concentrations of LTB_4 were the cause or product of neutrophil infiltration. It is suggested that the rapid metabolism of LTB_4 at inflammatory sites may serve to localize and limit its duration of action (Simmons *et al.*, 1983). Indeed, this was demonstrated in mares with persistent endometritis in which intra-uterine concentrations of LTB_4 were not significantly elevated, even though concentrations of prostaglandins were significantly higher than in genitally normal mares (Watson *et al.*, 1987b).

Leukotriene B_4 has been shown to have a possible role in the regulation of leucocyte function other than as a chemotaxin. *In vitro* techniques have shown that LTB_4 induces suppressor cells in assays studying mitogen-induced bovine lymphocyte transformation (Atluru & Goodwin, 1986) and also enhances complement receptors on human neutrophils and eosinophils (Nagy *et al.*, 1982). However, results from the present study would suggest that LTB_4 is not playing an important part in the pathogenesis of endometritis in susceptible mares as intra-uterine concentrations were not significantly different from those in genitally normal mares.

Concentrations of prostaglandins in uterine washings after experimental induction of endometritis were significantly affected by ovarian steroids (Watson *et al.*, 1987c) whereas there was no apparent effect on concentrations of LTB_4. Other studies have suggested that the apparent suppression of neutrophil migration into the uterus of mice and ewes during periods of progesterone domination may be due to inhibition of chemotactic substances, such as arachidonate metabolites (Staples *et al.*, 1983; Finn & Pope, 1986). In the present study, however, there was no evidence that, during the acute phase of neutrophil infiltration, progesterone inhibited synthesis of the most potent chemotactic arachidonate metabolite, LTB_4.

In conclusion, leukotriene B_4 was synthesized in response to induction of acute endometritis but it is not known whether it was a cause or result of the massive neutrophil infiltration.

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NEUTROPHIL CHEMOTAXIS IN THE HORSE IS NOT MEDIATED BY A COMPLEX OF EQUINE NEUTROPHIL ELASTASE AND EQUINE ALPHA-1-PROTEINASE INHIBITOR

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SUMMARY

Studies have demonstrated that as a result of proteolytic inactivation or complex formation (with neutrophil elastase), human alpha-1-proteinase inhibitor (API) becomes a potent chemoattractant for human neutrophils. The present study aimed to investigate the *in vitro* chemotactic response of equine neutrophils to an equivalent complex of equine API and neutrophil elastase. No evidence of neutrophil migration was observed towards purified complex derived from equine neutrophil elastase and the Spi 1 isoform of equine API, or to crude mixtures of porcine pancreatic elastase and unseparated equine API isoforms, although the same neutrophil preparations actively migrated towards zymosan activated plasma. It was concluded that, in the horse, complexes of API are not involved in the migration of neutrophils to sites of inflammation.

INTRODUCTION

The clinical importance of human alpha-1-proteinase inhibitor (API) as an inhibitor of neutrophil elastase has been appreciated since the association in man between a deficiency of this serpin (serine proteinase inhibitor) and premature development of emphysema and chronic liver disease (Carrell, 1986). However, more recent studies have suggested that API also has a role as an inflammatory mediator. These studies have demonstrated that API, and other members of the human serpin family, either as a result of complex formation with their target proteinases or after proteolytic inactivation are potent chemoattractants *in vitro* for human neutrophils (Banda *et al.*, 1988a, b; Baran *et al.*, 1989; Potempa *et al.*, 1991a). The chemotactic activity has been localized to the carboxyl terminal cleavage fragment formed after hydrolysis of peptide bonds in the region of the reactive site (Banda *et al.*, 1988a).

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Less information is available about the functions and involvement of API in disease states in other species. In the horse, several isoforms of API have been identified with the Spi 1 isoform being the most potent inhibitor of equine neutrophil elastase and the nearest equivalent to human API (Patterson *et al.*, 1991; Potempa *et al.*, 1991b).

Extensive neutrophil infiltration is a feature of several equine inflammatory diseases, for example, endometritis (Kenney, 1978) and chronic obstructive pulmonary disease (Kaup *et al.*, 1990). Neutrophil chemotactic factors have been identified in lavage fluid from mares affected with endometritis but they have not been characterized (Blue *et al.*, 1984). We therefore investigated whether the complex formed between equine neutrophil elastase and equine API is chemotactic for equine neutrophils, in order to establish whether this complex might be involved in the development of inflammation in the horse.

MATERIALS AND METHODS

Purification of neutrophil elastase

Equine neutrophil elastase was purified from 4.5 l of peripheral blood (from one adult thoroughbred stallion) using a modification of the method described by Dubin *et al.* (1976). White blood cells (75% neutrophils) were obtained by centrifugation (600 ***g*** for 15 min) of plasma following sedimentation of erythrocytes. Remaining erythrocytes were removed by hypotonic lysis. The neutrophil pellet obtained was resuspended in 0.2 M sucrose containing 150 IU heparin/ml and then homogenized for 20 s (Polytron, Kinematica, Lucerne, Switzerland) to disrupt the cells. Cell debris was removed by centrifugation at 2000 ***g*** for 25 min. The supernatant was centrifuged at 20 000 ***g*** for 25 min producing a granule pellet. This pellet was washed twice in 0.3 M sucrose and finally resuspended in 1 M NaCl containing 0.05% Triton-X 100. The suspension was stored at −50°C at this stage.

After thawing, the suspension was stirred overnight at 4°C to disrupt the granules. The extract was then diluted 1:7 in 50 mM 2-[N-Morpholino] ethanesulphonic acid (MES) (pH 6). Elastase was separated from the extract by fast protein liquid chromatography (FPLC) using a Mono-S ion exchange column (Pharmacia LKB Biotechnology, Milton Keynes, UK) Buffer A=50 mM MES pH 6; Buffer B= 50 mM MES pH 6, 1 M NaCl). Fractions were collected and elastase activity, which eluted at 200 mM NaCl, was detected using the substrate CBZ-L-Alanine-p-nitrophenyl ester (Sigma Chemical Co., Poole, UK). Fractions containing active enzyme were visualized by SDS-polyacrylamide gel electrophoresis (SDS-PAGE), (Laemmli, 1970) (12% resolving gel) followed by staining with Coomassie Brilliant Blue (Sigma Chemical Co., Poole, UK). Estimates of protein concentration were made using the bicinchonic acid protein assay (Pierce and Warriner, Chester, UK). The fractions were then stored at −50°C.

Purification of alpha-1-proteinase inhibitor (API)

The Spi 1 isoform of API was purified with residual esterase contamination from equine serum (API phenotype LL) by the method of Potempa *et al.* (1991b). The

mixture was separated on an SDS–PAGE gel, and the Spi 1 band was excised and electro-eluted.

To enable batch purification of API from serum an affinity column was prepared. Antiserum to the eluted protein was raised in a sheep, and affinity purified on a column containing the Spi 1 protein as a ligand. The affinity-purified antibody preparation was linked to CNBr-activated Sepharose 4B, and packed as a column. Equine serum (API phenotype LL) was diluted with PBS, then passed down the column. The retained protein was eluted in 0.1 M citric acid; 0.5 M NaCl and the pH was restored to neutrality by the immediate addition of 1 M Tris.

Pooled API fractions were concentrated by ultrafiltration, buffer exchanged on Sephadex G25 to 5 mM phosphate; pH 6.5 (containing 0.01% NaN_3), then chromatographed, using a Mono-Q ion exchange column, in the same buffer. The Spi 1 inhibitor eluted as a sharp peak at 100 mM NaCl with minimal contamination when visualized by SDS–PAGE.

Purification of API-elastase complex

A reaction mixture containing approximately 1:1 mol/mol API:elastase was incubated at room temperature (25°C) for 5 min, then 3,4-dichloroisocoumarin (DCI) (0.1 mM) was added to inhibit residual elastase activity. The mixture was first diluted 1:2 in PBS and 1:5 in 5 mM phosphate buffer (pH 6.5, 0.0001% NaN_3). The complex was separated using Mono-Q FPLC (Buffer A=5 mM phosphate, 0.0001% NaN_3; Buffer B=5 mM phosphate, 1 M NaCl, 0.0001% NaN_3). Fractions were collected and the complex identified using SDS–PAGE. Protein concentration was estimated from the absorbance at 280 nm. Fractions were stored at −50°C.

Neutrophil preparation

Neutrophils for the chemotaxis assay were separated from peripheral equine blood collected in EDTA from two donor animals. Following erythrocyte sedimentation, the plasma was layered on to Histopaque (density 1.09 g/ml) (Sigma Chemical Co., Poole, UK) and centrifuged at 200 *g* for 10 min. The Histopaque layer containing the neutrophils was separated and centrifuged at 2000 *g* for 5 min. The pellet was washed twice in PBS, erythrocytes were removed by hypotonic lysis and the pellet was washed once more in PBS. The neutrophils were then resuspended in 1×HBSS+1% BSA+4 mM bicarbonate at a concentration of 2×10^6 neutrophils/ml (>94% neutrophils, >99% cells excluded Trypan blue dye).

Chemotaxis assay

A modified Boyden chamber type assay (Boyden, 1962) was used to assess neutrophil chemotaxis. The chamber consisted of two 1.5 ml Eppendorf tube caps (Eppendorf, Hamburg, Germany) separated by a mixed cellulose ester filter, pore diameter 3 μm (Millipore, Watford, UK). Neutrophils were placed in the upper chamber and chemoattractants in the lower chamber. Chemoattractants that were compared were dilutions of the purified complex in media (1×HBSS containing 1% BSA and 4 mM bicarbonate), medium alone and zymosan activated plasma (Sedgwick *et al.*, 1987). The chambers were incubated at 37°C in a humidified atmosphere of 5% CO_2 for 45 min. The filters were removed, fixed in 98% etha-

nol for 20 min, stained with haematoxylin and cleared in xylene overnight. Ten fields from each filter were then examined at × 400 magnification and neutrophil migration was estimated using the leading front method (Zigmond & Hirsch, 1973). Chambers were run in triplicate and the results presented are the mean± SEM for the distance migrated by the neutrophils in three filters. Values were compared using the Students *t*-test.

RESULTS

The Spi 1 isoform of API (MW 55 000 estimated from SDS–PAGE) was isolated at a purity of >90% using the two step procedure described (Fig. 1). The isolated API isoform did not induce neutrophil migration at concentrations of 10^{-7}–10^{-9} M.

The fractions isolated from equine neutrophils which showed enzyme activity, as demonstrated by proteolysis of CBZ-L-Ala-NPE substrate, contained a 31 000 MW protein consistent with the type 2A elastase previously isolated from horse neutrophils (Dubin *et al.*, 1976). These fractions also consistently included higher molecular weight contaminants in addition to the elastase so it was decided to purify the elastase/API complex directly as described in the method section for use in the subsequent chemotaxis assays.

Purification of the reaction mixture containing 1:1 (mol:mol) API:neutrophil elastase resulted in the isolation of a protein complex of approximately 78 000 MW which was revealed as a single band on SDS–PAGE (Fig. 1). Dilutions of this complex were used to assess chemotactic activity in two experiments.

The distance moved by the leading front of the neutrophils towards zymosan activated plasma was significantly ($P<0.001$) greater than towards medium alone in both experiments. In experiment 1 (Fig. 2), although neutrophils showed a tendency to migrate less towards dilutions of the complex ranging from 10^{-8} to

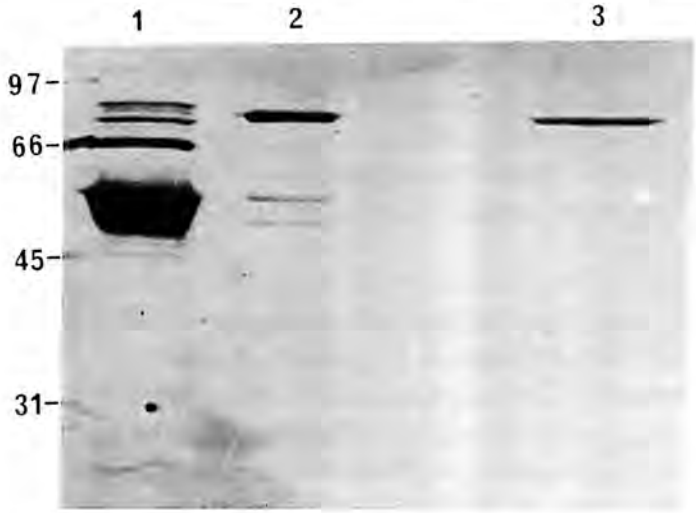

Fig. 1 Silver stained SDS–PAGE gel. Lane 1, Spi isoform of equine API; lane 2, reaction mixture at time 0 containing 1:1 (mol:mol) Spi 1 isoform of API: neutrophil elastase; lane 3, Mono-Q purified API/elastase complex.

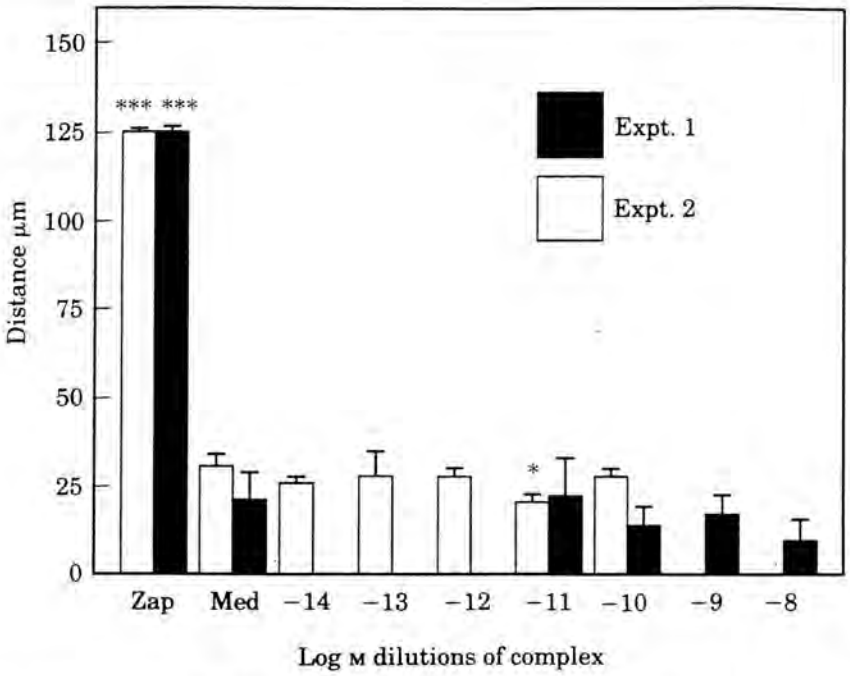

Fig. 2 Distance (mean±SEM) migrated by equine neutrophils in response to zymosan activated plasma (Zap), medium alone (Med) and dilutions of API/elastase complex in two experiments. (*P<0.05, ***P<0.001 significantly different from medium alone).

10^{-11} M compared to the distance moved towards medium alone, this was not statistically significant. In the second experiment (Fig. 2), there was a significant (P< 0.025) reduction in neutrophil migration towards 10^{-11} M complex compared to medium alone but this was not repeated at the other higher or lower concentrations (10^{-10}–10^{-14} M).

Two further experiments were performed using Boyden type chambers and unpurified mixtures of approximately 1:1 (mol:mol) ratios of porcine pancreatic elastase and either the Spi 1 isoform of API or crude preparations of unseparated API isoforms diluted in media (Table I). Although complex formation was confirmed by SDS–PAGE in these experiments, there was no significant difference in neutrophil migration towards dilutions of these mixtures compared to medium alone.

DISCUSSION

The results of this study indicate that in the horse the complex formed between API and neutrophil elastase is not chemotactic for equine neutrophils at the range of concentrations studied (10^{-8}–10^{-14} M). This range of concentrations was chosen because previous studies had shown that the maximum chemotactic response of human neutrophils occurred at a concentration of 10^{-9} M for complexes of both human API and human alpha-1-Anti-chymotrypsin (Banda *et al.*, 1988b; Potempa *et al.*, 1991a). The lack of neutrophil migration to the crude mixtures of API and porcine pancreatic elastase supports the results obtained using the purified com-

Table I

Mean±SEM distance (mm) migrated by equine neutrophils towards zymosan activated plasma (Zap) or dilutions of porcine pancreatic elastase and either Spi 1 isoform of API (experiment 3) or mixed API isoforms (experiment 4)

	Zap	*Medium alone*	*M concentrations of elastase/API*						
			10^{-12}	10^{-11}	10^{-10}	10^{-9}	10^{-8}	10^{-7}	10^{-6}
Expt. 3	113.9±6.7*	47.1±2.7	—	—	52.1±5.2	42.5±0.8	44.8±0.8	44.8±1.2	42.2±2.2
Expt. 4[a]	97.8±1.7†	17.9±2.4	16.9±1.2	17.2±0.8	20.2±1.2	19.2±1.0	15.0±1.6	14.3±1.2	—

*$P<0.01$; †$P<0.001$ significantly different from medium alone; [a]30 min incubation time.

plex and suggests that the other isoforms of equine API also would not stimulate neutrophil chemotaxis.

The generation of chemotactic activity following proteolytic cleavage or complex formation appears to be a general property of human serpins and has been shown to occur for API, α-1-Anti-chymotrypsin, C1 inhibitor and antithrombin III (Potempa *et al.*, 1991a). A pentapeptide sequence on the carboxyl side of the reactive site (residues 370–374) has been implicated in the receptor recognition of human serpin complexes and cleaved inhibitors by human monocytes and hepatoma cells (Perlmutter *et al.*, 1990; Joslin *et al.*, 1991). It has been hypothesized that this sequence may also be involved in neutrophil chemotaxis (Potempa *et al.*, 1991a) as the region of the reactive site is highly conserved in human serpin molecules. There is also 59% homology between the published sequence for the reactive site of equine API (Spi 1) (Patterson *et al.*, 1991) and human API. The corresponding equine API pentapeptide domain has an N-terminal phenylalanine and a carboxyl terminal isoleucine residue, features which were found to be essential for receptor recognition of the human API (Joslin *et al.*, 1991). This apparent species difference in chemotactic activity may therefore be due to a difference in neutrophil receptor populations.

The diversity of response of neutrophils from several mammals to the chemotactic peptide N-formyl-L-methionyl-L-leucyl-L-phenylalanine (FMLP) sets a precedent for this theory. FMLP is a chemoattractant for human, rabbit and guinea pig neutrophils. However, pig neutrophils have no receptors for the peptide and do not show a chemotactic response (Chenoweth *et al.*, 1980). Equine neutrophils, despite having high affinity receptors for FMLP (Snyderman & Pike, 1980), show chemotactic responses only at comparatively high molarities (10^{-4}) (Camp & Leid; 1982; Zinkl & Brown, 1982). It is possible that a chemotactic response might be elicited by higher concentrations of the equine complex than were available for the current experiment.

The biological relevance of the range of concentrations of complex used in this experiment and in the previous studies on human neutrophils (Banda *et al.*, 1988a, b); Baran *et al.*, 1989; Potempa *et al.*, 1991a) requires to be clarified. Alpha-1-proteinase concentrations have been estimated at 10^{-6} M in human alveolar lining fluid (Wewers *et al.*, 1987) and 10^{-7} M in normal equine uterine fluid (unpublished observations) but as yet no data have been obtained on the concentrations of complex in either tissues or biological fluids. Clearly more work is

required to establish whether the chemotactic effect observed in studies of human neutrophils *in vitro* is relevant *in vivo*.

In conclusion, this study provides no evidence in the horse that complexes of API and elastase are involved, via the stimulation of neutrophil chemotaxis in the inflammatory process.

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EQUINE VETERINARY JOURNAL
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Opsonins in uterine washings influencing *in vitro* activity of equine neutrophils

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Summary

Uterine washings were found to promote neutrophil mediated killing of *Streptococcus zooepidemicus*. Depletion of complement and/or specific antibody from the washings significantly reduced bactericidal activity. Phagocytosis of yeast by uterine washings was complement dependent. Inhibition of the classical pathway significantly reduced opsonic activity indicating that, in addition to direct activation via the alternate pathway, antibody may also be involved in yeast phagocytosis.

Introduction

IN the genitally-normal mare, fast and effective elimination of bacteria introduced at service or parturition requires efficient interaction between humoral and cellular defence mechanisms (Asbury *et al* 1982). The components in uterine secretions which promote bactericidal activity of neutrophils have not been studied in detail. A recent report has indicated that IgG may be involved (Hansen and Asbury 1987) and, in the presence of serum and some uterine flushings, phagocytosis of *Streptococcus zooepidemicus* appeared to be complement-dependent (Asbury, Gorman and Foster 1984; Brown, Hansen and Asbury 1985).

The purpose of the present study was to investigate the role of specific antibody and complement in uterine secretions in promotion of neutrophil phagocytic and bactericidal activities.

Materials and methods

Animals and sample collection

Two ovariectomised and two seasonally anoestrous mares which had no evidence of any pathological changes on histological examination of endometrial biopsies were used. Serial uterine washings were performed over seven days using 40 ml sterile phosphate buffered saline (PBS; 0.1M; pH 7.0). The washings were kept on ice until centrifuged at 10,000 *g* for 30 mins at 4°C. Aliquots of the supernatant were stored at –70°C until use. All treatments were performed using a total of 12 washings from the four mares, unless otherwise stated.

Bactericidal assay

The assay, along with isolation of blood neutrophils, was performed as previously described (Watson *et al* 1987a) using 0.1 ml *S zooepidemicus* (3 x 10^6/ml), 0.2 ml neutrophils (1 x 10^7/ml) collected from a gelding and 0.3 ml uterine washings. The tubes were prepared in duplicate and rolled at 37°C for 2 h. Aliquots (0.02 ml) were incubated overnight at 37°C on blood agar plates and the number of colony forming units counted. Bacterial survival was calculated as a percentage of the survival in duplicate tubes in which neutrophils were omitted and uterine washings replaced by 1 per cent horse serum.

Treatment of washings

Protein concentration. – Total protein was measured colorimetrically in washings using a Microprotein Rapid Stat Kit (Clandon Scientific; Aldershot). Washings were standardised for protein concentrations by diluting all samples to that of the lowest sample (20 mg/litre). The maximum dilution required was 10-fold. The washings were then used as opsonins in the bactericidal assay.

To study the effect of protein concentration on bacterial survival, human serum albumin was added to PBS to give concentrations of 0.1 to 100 mg/litre. In the absence of neutrophils, bacteria (0.1 ml, 3 x 10^6/ml) were added to the protein solutions (0.3 ml), rolled for 2 h at 37°C and diluted and plated out as described for the bactericidal assay.

Heat treatment. Washings were heated to 56°C for 30 mins to inactivate complement.

Yeast treatment. – Baker's yeast was prepared by boiling as previously described (Soothill and Harvey 1976). Yeast (1 x 10^8 blastospores/ml; 0.05 ml) was added to washings (0.6 ml) in duplicate stoppered plastic tubes and rolled at 37°C for 1 h. The tubes were then centrifuged at 2000 *g* for 10 mins and the supernatant removed. This process was repeated with supernatant from one of each pair of tubes. This process activated the alternative pathway of complement and removed C3.

Bacterial treatment. – A genital strain of *S zooepidemicus* was grown overnight in Brain Heart Infusion Broth, washed twice in PBS and resuspended at 1 x 10^7/ml by calibration of optical density with a spectrophotometer. Washings (0.6 ml) were added to bacteria (0.02 ml) and rolled at 37°C for 1 h. The bacteria were removed using millipore filters (0.45 µm; Flowpore D26; Flow Laboratories, Irvine) and the treatment repeated. This process removed antibody specific for *S zooepidemicus* and some C3 by activation of complement.

In a further experiment, the washings were treated once with yeast for 1 h followed by incubation with bacteria for 1 h and prepared for use in the bactericidal assay as described above.

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Titration with PBS. – A washing was serially diluted to 1:1000 and the dilutions were used in the bactericidal assay.

In six washings, neutrophils were substituted by 0.2 ml HEPES buffered Hanks Balanced Salt Solution (Flow Laboratories) and the bactericidal activity compared with that obtained in the presence of neutrophils.

Yeast opsonisation by uterine washings

The assay was performed as described previously (Soothill and Harvey 1976) with minor modifications. Blood neutrophils were isolated (Watson *et al* 1987b) and 1 x 10^8 heat killed yeast blastospores/ml (0.2 ml) were added to 0.1 ml washing and 0.1 ml neutrophils (1 x 10^7/ml). Tubes were rolled for 45 mins at 37°C and then centrifuged at 50 *g* for 5 mins. The sediment was resuspended in saline and a cytospin preparation was stained with Giemsa. The number of blastospores ingested by 100 neutrophils was counted and results expressed as blastospores/neutrophil.

Factors involved in opsonisation of yeast blastospores

The importance of complement in phagocytosis of yeast by neutrophils was assessed by heat treatment of three washings at 56°C for 30 mins to inactivate complement.

The relative contributions of the alternative and classical pathways of complement were investigated as described by Turner and others (1985) by pre-incubating four uterine washings with equal volumes of either PBS (pH 7.4) or 0.01M Mg EGTA (prepared by mixing equal volumes of 0.02M magnesium chloride and 0.02M EGTA dissolved in barbitone buffered saline) for 15 and 60 mins at 37°C. The washings were then used as opsonins in the yeast phagocytosis assay.

Statistical analysis

Results were analysed by a paired *t* test and correlations were tested by Pearson's correlation test. All means are quoted ± sem. Results were considered significant when P <0.05.

Results

The results of the different treatments performed on washings are shown in Table 1. Standardisation of washings for protein concentrations (20 mg/litre) had no effect on bacterial survival compared with undiluted washings and there was a direct correlation between bacterial survival in protein corrected and undiluted washings (r = 0.688; P <0.02). In the absence of neutrophils, the addition of a range of concentrations of human serum albumin to PBS did not significantly affect bacterial survival ($\bar{x}$ = 104 ± 5.1 per cent).

Increased bacterial survival was found in the bactericidal assay in which washings had been heat-treated or pre-treated with yeast and/or bacteria. The largest effects occurred after heat-treatment, repeated yeast pre-treatment and combined yeast and bacterial pre-treatment (Table 1).

TABLE 1: Effect of pre-treatment of uterine washings on their ability to promote bactericidal activity by neutrophils

Treatment	% mean ± sem bacterial survival Treated washings	Untreated washings	Level of significance
Protein corrected	52.8 ± 9.57	42.3 ± 8.65	NS
Heat treated	69.1 ± 11.33	25.1 ± 7.25	P <0.01
Yeast 1 x	43.5 ± 8.74	25.1 ± 7.25	P <0.01
Yeast 2 x	62.3 ± 4.85	25.1 ± 7.25	P <0.01
Yeast + *S zooepidemicus*	69.6 ± 6.70	25.1 ± 7.25	P <0.01
S zooepidemicus 2x	43.8 ± 8.46	25.1 ± 7.25	P <0.05

NS not significant

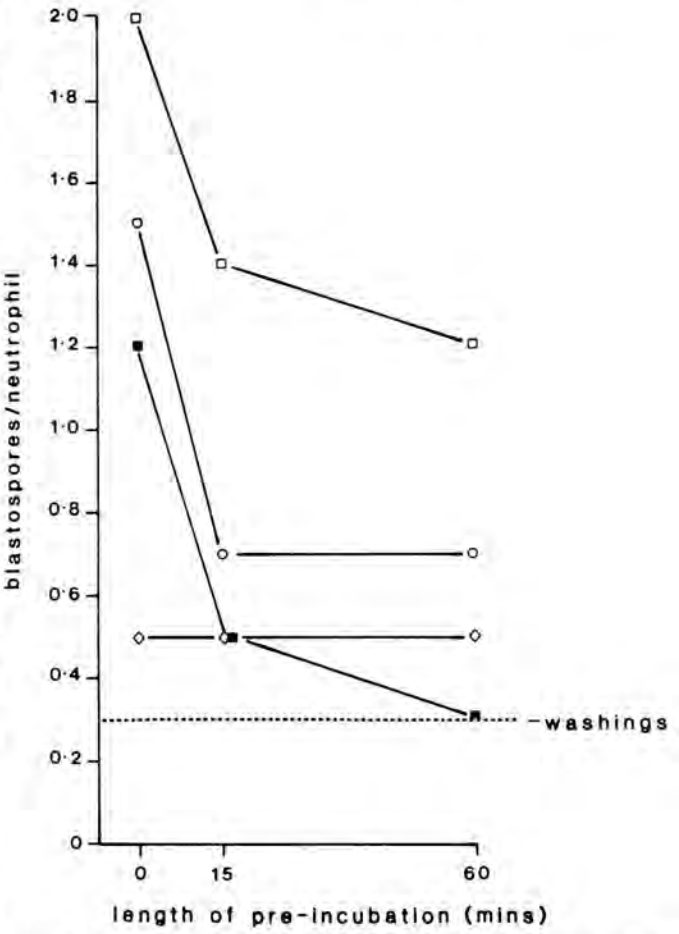

Fig 1. Effect of blocking the classical pathway of complement on opsonisation of yeast by uterine washings. Washings were mixed with PBS (time 0) or pre-incubated with a buffer containing 0.01M Mg EGTA for 15 or 60 mins. Each solid line represents a single uterine washing. The broken line represents tubes in which washings were replaced by PBS

Titration of washings with PBS resulted in a marked increase in bacterial survival between dilutions of 1:50 (16 per cent) and 1:1000 (47 per cent). Using the undiluted washing, bacterial survival was 14 per cent.

Omission of neutrophils from the assay resulted in a significant increase (P <0.02) in bacterial survival in the presence of uterine washings (84 ± 4.9 per cent) compared with replicate tubes containing neutrophils (47 ± 9.9 per cent).

Mean (± sem) number of blastospores ingested per neutrophil in the presence of untreated washings was 1.8 ± 0.46 but in the presence of heat treated washings was 0.6 ± 0.03. In two control tubes in which washings were substituted by PBS, number of blastospores per neutrophil was 0.6. The effect on yeast phagocytosis of abolishing classical pathway activation is shown in Fig 1. After incubation with Mg EGTA for 15 or 60 mins, there was markedly reduced phagocytosis except with the washing with initially low opsonising potential.

Discussion

In the present study, removal of either complement or specific antibody from uterine secretions resulted in a marked decrease in the bactericidal activity of neutrophils suspended in those secretions. The presence of both titres of specific antibody to *S zooepidemicus* and haemolytic complement activity have

already been demonstrated in uterine washings (Watson 1987; Watson, Stokes and Bourne 1987c). Non-specific opsonins and substances which stimulate neutrophil activity may have contributed to the residual opsonising capacity of washings which remained after treatment. Maximum bactericidal activity occurred only when components of both the classical and alternative pathways of complement were intact. Standardisation of protein concentrations in washings did not affect bactericidal activity and dilutions of greater than 1:50 were necessary before bacterial killing decreased. This would indicate that the components necessary for bactericidal activity were present in excess of the dilution factors necessary for standardisation of protein concentrations between washings.

Washings apparently possessed little inherent ability to kill bacteria in the absence of neutrophils. Other workers have shown that washings collected during dioestrus exhibited significant bactericidal activity (Strzemienski, Do and Kenney 1984), but the same group was unable to demonstrate this activity in washings collected from ovariectomised mares (P. J. Strzemienski, personal communication). Thus it appears that secretions from cycling intact mares may differ qualitatively from those of ovariectomised mares, perhaps due to the absence of hormonal priming.

Opsonisation of yeast blastospores by uterine washings was complement-dependent. Baker's yeast has been used in many studies of opsonic properties of serum (Soothill and Harvey 1976; Levinsky, Harvey and Paleja 1978; Turner *et al* 1986). Defective opsonic function of serum is associated with suboptimal deposition of C3b/C3bi:opsonin (Turner, Mowbray and Robertson 1981) resulting from the inactivity or absence of a cofactor that is required for C3b deposition (Turner *et al* 1985). In the absence of specific antibody, yeast will activate complement via the alternative pathway (Soothill and Harvey 1976; Turner *et al* 1976). However, using human serum, it was demonstrated that antibody may be present which permits the classical pathway to contribute to complement activation (Turner *et al* 1985). Results from the present study on neutrophil phagocytosis of yeast blastospores suspended in uterine washings, similarly showed a contribution from the classical pathway. It seems likely that either non-specific opsonins or cross-reacting antibodies are present in washings which were produced initially against other common yeast or bacterial cell wall antigens.

In conclusion, phagocytic and bactericidal activity of neutrophils in uterine washings was both complement- and antibody-dependent.

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Sequelae of myelography in the horse

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Summary

The records of 131 horses undergoing general anaesthesia and positive contrast cervical myelography with metrizamide were examined to determine the effect of the procedure on the 'patient'. Three per cent of minimally ataxic and moderately ataxic horses had serious complications after myelography. Thirty-two per cent of severely ataxic horses died or were destroyed after general anaesthesia and myelography. Although general anaesthesia and myelography are essential components of a complete neurological evaluation of a horse, they impose a significant risk.

Introduction

DEFINITIVE diagnosis of cervical spinal cord disease in the horse often requires positive contrast myelography. Despite a recent report of myelography in six standing horses (Foley, Gatlin and Selcer 1986), most myelography is performed on horses under general anaesthesia. The risks associated with general anaesthesia are well known. The previously described complications associated with myelography and general anaesthesia include fever, depression, anaphylaxis, stiffness of the neck, seizures, muscle twitching, increases in muscle tone, and transiently worsened ataxia (Stowater, Kneller and Froelich 1978; Beech 1979; Nyland *et al* 1980; Rantanen, Gavin, Barbee and Sand 1981; Conrad 1984). The majority of complications that have been associated with myelography and general anaesthesia are transient, but occasionally myelography has altered the outcome of the disease process (Conrad 1984). The purpose of this paper is to review the sequelae of myelography and general anaesthesia in a series of 131 horses with graded neurological disease.

Materials and methods

Records of 131 horses that underwent general anaesthesia and myelography at the Ohio State University Veterinary Teaching Hospital over an eight year period were evaluated. Medical records were analysed to determine whether general anaesthesia and myelography had an effect on the outcome. Ataxia was graded by a system modelled on that developed by deLahunta (1977). This information is summarised in Table 1.

Pre-anaesthetic procedures varied with the clinician. Survey radiographs were either taken while the horse was tranquillised 24 to 48 h prior to myelography or under anaesthesia immediately prior to myelography. Horses were placed in lateral recumbency following the induction of general anaesthesia. A lateral plain radiograph in a neutral position was taken to optimise radiographic technique. The head was elevated approximately 0.6 m off the floor as described by Rantanen *et al* (1981). Thirty-five to 40 ml of cerebrospinal fluid was removed from the subarachnoid space at the atlanto-occipital junction following percutaneous placement of a 12.4 mm diameter 6 cm spinal needle. Analytical grade metrizamide was dissolved in sterile water and drawn through a 0.2 µm millipore filter into a sterile syringe. Metrizamide (180 mg/ml, 35 to 40 ml) was injected over a 2 to 4 min period. The head was allowed to remain elevated for 5 mins after the completion of injection. Standard radiographic views included three lateral views (cranial cervical, middle cervical and caudal cervical) with the neck in a neutral position, two lateral views (middle cervical and caudal cervical) with the neck in a hyperextended position, and three lateral views (cranial cervical, middle cervical and caudal cervical) with the neck in a flexed position. Additional views were taken when necessary to assure adequate evaluation of any suspicious areas.

Results

Breed, sex, age and weight

Eighty-two horses were Thoroughbreds (62 per cent). Other affected breeds were 19 Standardbreds (14.5 per cent), 10 Arabians (7.6 per cent), seven Quarterhorses (5.3 per cent), six Saddlebreds (4.6 per cent), and seven others (5.3 per cent). Seventy-six horses were stallions (58 per cent), 33 were females (25 per cent) and 22 were castrated males (16 per cent). The age at examination ranged from two months to 15 years with an average age of 1.9 ± 1.9 years. Horses averaged 365 ± 96.7 kg bodyweight.

Neurological deficit

Sixty-three horses (48 per cent) were classified as having grade 3 ataxia prior to myelography. Thirty-one horses (24 per cent) had grade 2 ataxia, 25 (19 per cent) had grade 4 ataxia, 10 (7 per cent) had grade 1 ataxia, and one horse was graded normal (grade 0). One horse was recumbent (grade 5).

TABLE 1: Classification of ataxia

Grade	Description
0	No gait deficits
1	Gait deficits barely perceptible, worse when head is elevated
2	Gait deficits noted at a walk
3	Gait deficits noted at rest and walking. Horse nearly falls when head is elevated
4	Horse falls or nearly falls at normal gaits
5	Recumbent patient

EFFECT OF SUSCEPTIBILITY TO ENDOMETRITIS ON SPECIFIC ANTIBODY IN THE ENDOMETRIA OF MARES

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ABSTRACT

Endometrial biopsies were collected on two occasions from mares resistant to (n = 3) and once from mares susceptible to persistent endometritis (n = 6). The endometrial tissue was minced and cultured in vitro for 24 h. No hemolytic complement activity was detected in the endometrial culture supernatant. Endometrial culture supernatant from mares with persistent endometritis contained titers of antibodies to Streptococcus zooepidemicus similar to those from resistant mares. However, the culture supernatant of biopsies from mares with endometritis was less effective ($P < 0.05$) at opsonizing S. zooepidemicus in vitro.

Key words: mare, endometritis, specific antibody

INTRODUCTION

The persistence of intrauterine infection in mares is a common cause of subfertility (1). Susceptible mares become infected at parturition, or during breeding or veterinary uterine examination and, instead of rapidly eliminating invading microorganisms, remain infected and do not conceive (2).

The most common pathogen isolated from cases of endometritis is S. zooepidemicus (3). Resistance to intrauterine infection requires an efficient neutrophil response, and although mobilization of neutrophils appears adequate in susceptible mares (4,5), phagocytosis by uterine neutrophils may be defective (6,7). The humoral immune system constitutes another important uterine defense mechanism. In most species, the systemic immune system is the major source of immunoglobulins in genital tract secretions. In the mare, it appears that either local synthesis of immunoglobulins or active transport from serum is more important than passive diffusion (8). Concentrations of immunoglobulins in uterine secretions from mares vary during the estrous cycle (9-11), and hormone treatment of ovariectomized mares affects endometrial antibody content (12). Mares susceptible to persistent endometritis have higher concentrations of immunoglobulins present

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in uterine secretions than normal mares (9,13,14). This finding has cast doubt on the functional capacity of immunoglobulins in the uterine secretions of these mares, but antibody titers to infective organisms have not been measured to date.

In the present study, we measured antibody response after intrauterine inoculation of S. zooepidemicus. Endometrial antibody content was assessed using in vitro culture of tissue from resistant mares and from mares with persistent endometritis. The capacity of opsonins in both serum and endometrial culture supernatant to opsonize S. zooepidemicus was evaluated.

MATERIALS AND METHODS

Animals

Three ovariectomized mares were used which had no evidence of any pathological changes on histological examination of endometrial biopsies. These mares were being used in a related study on the effect of ovarian steroids on uterine antibody content (12) and had been shown to be resistant to intrauterine infection with S. zooepidemicus. Six other mares with intrauterine infections of S. zooepidemicus were studied. These mares were classified as having persistent endometritis and had a history of subfertility. Examination of endometrial biopsies revealed Category III endometria by the criteria of Kenney (15).

In Vitro Release of Immunoglobulins from Endometria

The procedure was modified from a method described previously (16). Biopsies were collected into serum-free incubation medium (RPMI 1640, penicillin/streptomycin 5,000 units/ml; L-glutamine 200 mM/l; pH 7.3) and kept on ice. The biopsies were dissected with a scalpel into approximately 2-mm pieces. The fragments were weighed and 300 mg of tissue was suspended in 120 ml of medium in a flat-bottomed incubation flask[a] and incubated at 37°C in an atmosphere of 95% air : 5% CO_2. After 24 h, the flask was frozen at -20°C. When the tissues were thawed, the resultant disruption of cells released the endogenous immunoglobulins. The endometrial culture supernatants were stored in aliquots at -70°C.

Preparation of S. zooepidemicus Antigen

Streptococcus zooepidemicus was inoculated into 1 l of brain-heart infusion broth and incubated for 24 h at 37°C in an atmosphere of 95% air : 5% CO_2. The broth was centrifuged at 10,000 x g for 15 min to pellet the bacteria. The bacteria were then washed three times in sterile phosphate buffered saline (PBS, pH 7.3). The bacteria were resuspended in PBS and boiled in a water bath for 1 h. After washing the bacteria three times with 15 ml carbonate buffer (pH 9.6), the supernatants were pooled.

Quantitation of Specific Antibodies to S. zooepidemicus

Specific IgG, IgM and IgA antibodies were measured by an indirect enzyme linked immunosorbent assay (ELISA). All three classes were measured in endometrial culture supernatant and IgG and IgM in serum.

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[a] Titertek, Flow Laboratories, Irvine, UK.

The assay was performed using methods described previously (12). Twelve five-fold dilutions of serum, and six five-fold dilutions of culture supernatant were placed individually in wells which had been coated with S. zooepidemicus antigens. A positive standard (serum) was included in each assay. The antisera used were either affinity-purified sheep antisera (prepared in the University of Bristol School of Veterinary Science) to equine IgG or equine IgA, or rabbit anti-equine IgM.[b] These antisera were shown to be monospecific for the heavy chain of their respective isotypes. Alkaline phosphatase conjugates of pig antiserum to sheep IgG or sheep antiserum to rabbit IgG were added to each well. The substrate[c] was added after a final wash. Color was allowed to develop and the plates were read on a Titertek multiscan MC[d] using the dual wavelength mode at 405 and 494 nm. Background readings were obtained by the substitution of incubation buffer in individual wells for the primary antiserum, the sample, and the antigen coating, respectively.

Results were expressed as optical density for all portions of the experiment which permitted assays to be performed on the same day. Otherwise, the titers were calculated relative to the positive standard and presented as sample titer/standard titer x 100%.

Bactericidal Assay

This was performed as described previously (17) using heat-treated (56°C for 30 min) endometrial culture supernatant or 10% heat-treated horse serum as the opsonin (0.3 ml) for S. zooepidemicus (3×10^6/ml; 0.1 ml). Neutrophils (1×10^7/ml; 0.2 ml) from a gelding were added and the tubes were incubated on a roller at 37°C for 2 h. Triplicate drops (0.02 ml) of serial dilutions of the suspension were then placed on blood agar plates and incubated overnight at 37°C. The surviving bacteria were quantitated by counting the number of colony-forming units. Results were expressed as a percentage of the bacteria surviving when endometrial culture supernatant was replaced by 1% horse serum during the assay, and the neutrophils by HEPES buffered Hanks balanced salt solution (HBSS pH 7.3). Controls were included in which endometrial culture supernatant was added, but the neutrophils were replaced by HBSS and in which culture supernatant was replaced by medium. Before the endometrial culture supernatants were used in the assay, they were dialyzed overnight at 4°C against PBS pH 7.3 to remove antibiotics. The exclusion limit on the dialysis tubing was a molecular weight of 15,000.[e] Results used were the mean of two replicate assays performed on separate days.

Hemolytic Complement Assay

Porcine red blood cells were sensitized with a subagglutinating titer of rabbit anti-pig red blood cell serum and endometrial culture supernatant (0.3 ml) was added (18). The mixture was incubated at 37°C for 30 min. The degree of hemolysis was calculated by using a spectrophotometer at 541 nm and comparing the reading with a tube containing ammonia solution (0.04%) in which there was 100% hemolysis. Positive controls of serially diluted fresh horse serum were included. Negative controls were included in which the antiserum or endometrial culture supernatant was replaced with buffer.

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[b] Nordic Laboratories, Maidenhead, UK.
[c] Alkaline phosphatase substrate, Sigma, Poole, UK.
[d] Flow Laboratories, Irvine, UK.
[e] Medicell Int'l Ltd., London, UK.

Experimental Design

The three ovariectomized mares were treated daily for 14 d sequentially with intramuscular injections (4 ml) of estradiol benzoate (1 mg) or oily vehicle (arachis oil), with a period of 14 d between treatments. A previous study had shown that treatment with estradiol did not affect endometrial antibody content or opsonizing ability of culture supernatant (12). On Day 7 of both treatment periods, the mares were infected with live S. zooepidemicus (1×10^9) in 50 ml PBS by intrauterine infusion via a Foley catheter (24 F; 30-ml cuff). Endometrial biopsies were collected on Day 14. Endometrial swabs collected on Days 7, 10 and 14 were streaked onto blood agar plates which were then incubated overnight at 37°C. Blood samples were collected into evacuated tubes[f] on Days 7 and 14, and the serum was stored at -20°C. Uterine flushings were centrifuged at 2000 g and stored in aliquots at -20°C.

Endometrial biopsies were collected from four mares with persistent S. zooepidemicus infections. These mares were sampled in estrus or in winter anestrus based on previous data regarding the effect of estradiol on endometrial antibody content (12). Sera from these mares and from another two persistently infected mares were stored at -20°C.

Statistical Analyses

Results were analyzed by a Student's t-test.

RESULTS

When bacteria were opsonized with culture supernatant, omission of neutrophils resulted in 100% bacterial survival. Bacterial survival was 96% when unconditioned culture medium was added to bacteria in the presence of neutrophils. Opsonization of bacteria using culture supernatant from endometrial biopsies of susceptible mares resulted in lower bactericidal activity of neutrophils (54 ± 4.5%, $P < 0.05$) than supernatant from the biopsies of resistant mares (67 ± 3.9%). Opsonization of bacteria with serum from susceptible mares resulted in bactericidal activity (61 ± 8.1%) which was higher but not significantly different from that using serum from resistant mares (49 ± 4.1%). No hemolytic complement activity could be detected in any of the endometrial culture supernatants.

Titers of specific antibody to S. zooepidemicus in biopsy cultures from susceptible mares were compared with titers measured in culture supernatants from endometrial biopsies of resistant mares. Titers to S. zooepidemicus in IgG, IgM and IgA were similar in both groups of mares (Table 1).

Titers to S. zooepidemicus in IgG were not lower in serum of susceptible (OD = 1.0 ± 0.05) than in resistant (0.9 ± 0.03) mares. Similarly, titers to S. zooepidemicus in IgM were not lower in susceptible (OD = 0.5 ± 0.03) than in resistant (0.6 ± 0.03) mares.

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[f] Becton-Dickinson, Oxford, England.

DISCUSSION

Our study showed that although titers of antibody to S. zooepidemicus were similar in endometrial culture supernatant from susceptible and resistant mares, culture supernatant from susceptible mares was less effective at opsonizing bacteria than supernatant from resistant mares.

Previous studies have shown that treatment of ovariectomized mares with estradiol did not affect antibody titers or opsonizing capacity of endometrial culture supernatant (12). In our study, mares with persistent endometritis tended to have higher concentrations of antibodies in their endometria. High concentrations of IgG and IgA have been measured in the uterine secretions of mares with persistent endometritis (9,13,14), but antibodies to S. zooepidemicus have not been previously quantitated in uterine secretions.

Table 1. Titer (optical density at 405 and 494 nm) of antibody to S. zooepidemicus ($\bar{x} \pm$ SEM) in immunoglobulins present in culture supernatant of endometrial tissue from resistant and susceptible mares.

	# of mares	# of samples	Titer in Immunoglobulin		
			IgG	IgA	IgM
Resistant	3	6	0.466 ± 0.039	0.235 ± 0.021	0.734 ± 0.117
Susceptible	4	4	0.548 ± 0.049	0.266 ± 0.018	0.791 ± 0.062

Despite the apparently adequate concentrations of opsonizing antibody, the bactericidal activity of neutrophils was significantly lower when they were suspended in culture supernatant of endometria from mares with persistent endometritis than when suspended in culture supernatant of endometrial tissue from resistant mares. Not all subclasses of IgG act as opsonins. One subclass of IgG in horses, IgG_T, does not fix complement, does not mediate opsonization, and may inhibit complement fixation and opsonization by IgG_{ab} (19,20). Therefore, although titers of IgG antibodies in endometrial tissue may have been equivalent between groups, proportions of antibodies in IgG subclasses may have differed. By contrast, serum from susceptible mares tended to be more effective at opsonizing bacteria than serum from resistant mares, but this did not reach significance because of the variance in the six susceptible mares tested.

Despite the hemolytic activity previously measured in uterine flushings (18), no hemolytic complement activity could be detected in endometrial culture supernatant.

It is important that these results be extended to include a greater number of resistant and susceptible mares and to investigate possible deficiencies in uterine humoral and cellular interactions in opsonization and phagocytosis of bacteria.

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THERIOGENOLOGY

USE OF HYPERIMMUNE SERUM IN TREATMENT OF ENDOMETRITIS IN MARES

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ABSTRACT

Endometritis was induced in four progesterone-treated ovariectomized mares and in two seasonally anestrous mares by intrauterine infusion of Streptococcus zooepidemicus. The bacteria were suspended in phosphate buffered saline (PBS), or in nonimmune or hyperimmune serum. Uterine lavage was performed after 24 h. Significantly fewer ($P < 0.05$) bacteria were recovered from mares which received hyperimmune serum than from mares which received nonimmune serum or PBS. There was no significant difference between treatment with nonimmune serum or PBS. It is therefore suggested that increasing the availability of specific antibody reduced the severity of intrauterine infection.

Key words: endometritis, uterine defense, mare

INTRODUCTION

There is an almost linear drop in fertility with age in Thoroughbred mares(1). Many older, multiparous mares fail to resolve the acute endometritis induced by introduction of potential pathogens at coitus, and remain persistently infected(2,3). These so-called susceptible mares are thought to suffer from a deficiency in uterine immune defense mechanisms(4,5).

Uterine secretions from susceptible mares contain higher concentrations of immunoglobulins than secretions from genitally normal mares(6,7,8). Further, it has recently been shown that, after intrauterine infection of mares with Streptococcus zooepidemicus, endometrial explants from susceptible mares contained, and were capable of synthesizing, similar amounts of specific antibody as explants from resistant mares(9). However, when supernatants from these cultures were used to opsonize Str. zooepidemicus prior to phagocytosis by equine neutrophils, supernatant of cultures from susceptible mares were significantly less effective than those from resistant mares.

In this study we investigated the role of specific antibody in vivo in elimination of Str. zooepidemicus by using heat-treated hyperimmune serum to treat experimentally-induced endometritis. The cross-reactivity of the opsonins present in the hyperimmune serum was investigated in vitro with other bacteria.

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MATERIALS AND METHODS

Hyperimmune serum was raised against a genital strain of Str. zooepidemicus in a pony mare by three subcutaneous injections of 10^{10} heat-killed bacteria at intervals of three weeks. The vehicles for the consecutive inoculations were Freund's complete adjuvant, Freund's incomplete adjuvant and phosphate buffered saline (PBS; pH 7.3), respectively. The bacteria were prepared by overnight incubation at 37°C in Brain Heart Infusion Broth (BHIB). The bacteria were then washed twice in PBS and resuspended at 10^{10}/ml by calibration of optical density using a spectrophotometer.

The promotion of bactericidal activity of equine blood neutrophils (collected from a gelding) by nonimmune and hyperimmune heat-treated (56°C for 30 min) sera was measured in vitro using a procedure described previously (10). Briefly, blood neutrophils were prepared by hypotonic lysis and washed three times with PBS. Viable neutrophils, which excluded trypan blue were counted using a hemocytometer. The cells were diluted to a concentration of 1×10^7/ml. Eighteen-hour cultures of bacteria were washed twice in PBS and resuspended at 3×10^6/ml by calibration of optical density at 640 nm. Bacteria (0.1 ml) were added to neutrophils (0.2 ml) and 10% serum (0.3 ml) and incubated at 37°C for 2 h. All samples were performed in duplicate. At the end of the incubation, a portion was serially diluted to 10^{-3}. Triplicate aliquots (0.02 ml) were incubated at 37°C for 24 h on blood agar plates. Bacterial survival was calculated by the number of colony forming units obtained in tubes with serum/numbers of colony forming units in tubes in which serum was replaced by PBS x 100%. In addition to the equine genital strain of Str. zooepidemicus isolated from a mare with acute endometritis, strains of Str. uberis, Escherichia coli and Staphylococcus aureus, which had been isolated from the milk of cattle with mastitis, were used in the assay to test cross-reactivity of the sera.

The Str. zooepidemicus strain was isolated from a mare with acute endometritis. Aliquots were stored at -70°C in 10% glycerol in BHIB. A vial was thawed prior to use, inoculated into 10 ml BHIB and incubated overnight at 37°C. The bacteria were washed twice in PBS before being suspended at 10^9/ml by measurement of optical density at 640 nm.

Six pony mares with no histological evidence of endometritis, and that were either ovariectomized (n = 4) or seasonally anestrus (n = 2), were treated daily with progesterone[a] (100 mg) for 7 d. This dosage regimen produced plasma concentrations of progesterone similar to those measured during diestrus (9). On Day 6, the six mares were divided into three equal groups and received an intrauterine infusion of washed Str. zooepidemicus (10^9) suspended in either 100 ml of PBS (n = 2), nonimmune serum collected from a gelding (n = 2) or hyperimmune serum (n = 2). Both sera were heat-treated at 56°C for 30 min before use. Uterine lavage was performed using 40 ml PBS immediately before and 24 h after the infusion. Triplicate aliquots (0.02 ml) of dilutions of lavage fluid (to 10^{-4}) were incubated overnight at 37°C on blood agar plates and the number of colony forming units were counted. Neutrophils recovered in the lavage fluid were counted using a hemocytometer and viability was assessed by exclusion of Trypan Blue dye. The experiment was repeated three times in a Latin Square design so that each mare received all treatments, with a 14-d interval between experiments.

[a]Intervet, Cambridge, U.K.

Data from the bactericidal assay were analyzed using a two-tailed t-test. Bacterial survival in uterine flushings was compared using an ANOVA on $\log_{10}$ transformed data. Factors considered were mare and week of experiment. Differences between treatments were analyzed using a paired t-test. Correlations were analyzed using a Pearson's correlation test. All means are quoted ± SEM. Values of $P<0.05$ were considered significant.

RESULTS

In the absence of neutrophils, both nonimmune and hyperimmune sera lacked bactericidal activity in vitro. In the presence of neutrophils the hyperimmune serum was more effective ($P<0.001$) at opsonizing Str. zooepidemicus and Str. uberis than nonimmune serum (Table 1). Escherichia coli was relatively resistant to the bactericidal activity of neutrophils in the presence of either serum, and there was no significant difference between sera, whereas S. aureus was highly sensitive to neutrophil bactericidal activity (100% survived in the absence of serum).

TABLE 1. Mean[a] (± SEM) bactericidal activity of blood neutrophils suspended in nonimmune or hyperimmune serum

	Percent bacterial survival	
Bacteria added (3×10^6/ml)	Nonimmune serum	Hyperimmune serum
Streptococcus zooepidemicus	41 ± 2.0	16 ± 3.2
Streptococcus uberis	24 ± 3.0	7 ± 1.0
Escherichia coli	84 ± 4.7	77 ± 9.2
Staphylococcus aureus	0	0

[a] Mean of six replicates.

In uterine lavage fluid collected from mares after infusion of bacteria, there was no correlation between either the number of viable or dead neutrophils, or the number of colony forming units of Str. zooepidemicus present (Table 2). Between treatment groups there was no significant difference in the number of viable or dead neutrophils in lavage fluid,but fluid recovered from mares treated with hyperimmune serum contained significantly ($P<0.05$) fewer bacteria than fluid from mares treated with PBS or nonimmune serum. There was no significant difference in numbers of bacteria recovered from mares treated with nonimmune serum or PBS. Neither week of treatment nor mare had a significant effect on bacterial survival ($P = 0.70$ and $P = 0.57$, respectively).

TABLE 2. Mean (± SEM) numbers (log_{10}) of neutrophils and bacteria in lavage fluid from mares treated with intrauterine infusions of hyperimmune serum, nonimmune serum and PBS.

Number of neutrophils and bacteria in lavage fluid collected 24 h after bacterial infusion	Treatments		
	hyperimmune serum	nonimmune serum	PBS
Number of viable neutrophils/ml	6.9 ± 0.4	7.4 ± 0.3	7.0 ± 0.2
Number of dead neutrophils/ml	5.7 ± 1.2	7.2 ± 0.2	5.4 ± 1.1
Colony forming units/ml	3.5 ± 1.1	6.3 ± 1.0	7.4 ± 0.7

DISCUSSION

We investigated the capacity of specific opsonins in hyperimmune serum to combat infection with Str. zooepidemicus. To evaluate use of hyperimmune serum as a possible treatment for clinically affected mares, the cross-reactivity of the serum was tested in vitro with a range of other bacteria.

The hyperimmunization regimen boosted the opsonizing ability of serum for Str. uberis as well as Str. zooepidemicus in vitro. The presence of common cell wall antigens among streptococcal species has been described previously (11) and presumably explains the cross-reactivity of the serum. The strain of S. aureus used in our study (M60) is known to be highly sensitive to the bactericidal activity of neutrophils, even in the presence of very low concentrations of opsonins (12), whereas the capsulated E. coli (B 117) is highly resistant to phagocytosis (13). Thus opsonization by the sera varied with different bacteria and made it unlikely that the hyperimmune serum would have a superior effect to nonimmune serum in treatment of infections with certain bacteria.

Significantly fewer colony forming units of Str. zooepidemicus were recovered from mares infused with hyperimmune serum than with nonimmune serum or PBS. As the cellular response was similar in the three groups of mares, it is likely that the difference in the results could be attributed to the presence of opsonizing antibody or other less specific opsonins in the hyperimmune serum. It is known that progesterone treatment renders mares susceptible to uterine infection (14), possibly by reducing the amount of endometrial antibody available for bacterial opsonization (15). Our study illustrates, therefore, that by increasing antibody levels, infection can be reduced. However at 24 h, the infection was eliminated in only two of the mares treated with hyperimmune serum. The maximum cellular response is reached by 6 h after bacterial infusion (15), whereas in the our study approximately 50% of the neutrophils were dead by 24 h. It is possible that if the mares had been sampled earlier after introduction of infection, a more profound reduction in bacterial numbers would have been observed. Further, as the serum

possessed no inherent bactericidal activity, if the bacteria were not eliminated promptly, the serum retained within the uterus would provide a rich medium for bacterial multiplication.

By designing the experiment as a Latin Square it was possible to study the effect of repeated infusion on resistance to infection. There was no significant effect of repeated infection on the ability of the mare's uteri to clear infection, suggesting that the bacteria must either have acted as a very weak immunogen or the uterine immune response was very short-lived, with no evidence of immunological memory.

By employing sera which had previously been heat-inactivated, we did not investigate the role of complement in bactericidal activity within the uterus. Other studies have reported either no deficiency of opsonizing antibody or hemolytic complement in uterine secretions from susceptible mares (17) or the total absence of hemolytic complement from uterine secretions of mares (18). Provision of a source of complement, in the form of intrauterine infusion of plasma, has been advocated as a treatment for persistent endometritis (19). However, other workers have shown no significant benefit of plasma therapy (20).

In conclusion, the results from our study indicate that, although addition of specific antibody alleviated acute endometritis in part, other factors such as reduced phagocytic capacity of uterine neutrophils from progesterone-treated mares (21) may explain the failure to eliminate infection in four of the six mares treated with hyperimmune serum. A previous study has shown that immunization or repeated infection with the CEM organism, Taylorella equigenitalis, provided no protective response within mares despite elevated antibody titers in secretions. (22) Our results underline the significance of phagocytic involvement, apart from humoral components, in the protection of the genital tract.

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Macrophage clearance of ^{125}I-labelled polyvinyl pyrrolidone in the horse: Effect of ovarian steroids and persistent endometritis

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Summary

The rate of clearance of ^{125}I-labelled polyvinyl pyrrolidone (PVP) from blood was measured in mares as an indicator of macrophage function. In three out of four cycling mares, PVP clearance was slower during oestrus than dioestrus. Similarly, administration of oestrogen to four ovariectomised mares tended to depress PVP clearance compared with clearance from the same mares before they received oestrogen. However, the effect of oestrogen was not statistically significant. Mares susceptible to persistent endometritis had rates of PVP clearance which were similar to those of genitally normal mares.

Introduction

ENDOMETRITIS is an important cause of subfertility in the mare (Peterson, McFeely and David 1969; Hughes and Loy 1975). The progesterone-dominated uterus appears to be highly susceptible to endometritis, whereas during periods of oestrogen domination infection is quickly eliminated (Ganjam *et al* 1982). However, some mares are susceptible to uterine infection at all stages of the oestrous cycle and develop a persistent endometritis (Peterson *et al* 1969). Defects in both local antibody (Watson 1987) and in uterine phagocyte function (Cheung, Liu, Walsh and Miller 1985; Watson, Stokes and Bourne 1987a,b) have been detected in both of these susceptible groups of mares using *in vitro* tests. However, few studies have been performed in the living animal on specific components of the immune system under the influence of oestrogen or progesterone or in mares with persistent endometritis.

Macrophages operate a central role in both the afferent and efferent limbs of the immune system. They take up antigen, process it and present it to lymphocytes along with soluble factors which regulate the inductive phase of the immune response (Unanue, Beller, Lu and Allen 1984). Macrophages also play an important part in phagocytosis during bacterial infections and treatment of animals to block the activity of or to kill their macrophages, increases the infectivity or lethality of pathogens (O'Brien, Scher and Formal 1979). Within the reproductive tract, macrophages are present both within the uterine lumen and in the endometrium itself during periods of chronic inflammation (Kenney 1978; E. D. Watson unpublished data) and are presumably playing an active role in elimination of infection. The classical test for macrophage clearance in laboratory animals employs injection of large doses of carbon resulting in a dose-related blockade of macrophages (Benacerraf, Biozzi, Halpern and Stiffel 1957). More recently, clearance of ^{125}I-labelled polyvinyl pyrrolidone (PVP) has been validated as a method for assessing macrophage function *in vivo*. Decreased clearance rates after intravenous injection of PVP are associated with depressed macrophage function. This test has the advantage that it does not induce macrophage blockade and is therefore independent of dose (Morgan and Soothill 1975a)

The present study investigated clearance of PVP from mares under the influence of elevated plasma concentrations of oestradiol and progesterone and from mares with persistent endometritis.

Materials and methods

Four ovariectomised and four cycling pony mares, which had no evidence of endometritis on histological examination of biopsies, were studied. A further five mares, which had a history of recurrent and persistent endometritis and which showed evidence of polymorphonuclear and mononuclear cell infiltration, gland dilatation and fibrosis on histological examination of endometrial biopsies were also used.

PVP clearance was investigated both in the ovariectomised mares after seven daily intramuscular injections (4 ml) of oily vehicle or oestradial benzoate (1 mg; Intervet; Cambridge), and in cycling mares in mid-oestrus and in mid-dioestrus (seven to 10 days post ovulation). Mares with persistent endometritis were studied during an oestrous period which had been induced by a prostaglandin injection (Lutalyse; Upjohn, Crawley) during dioestrus. ^{125}I-PVP (Amersham, High Wycombe) was injected in 2 ml saline into the jugular vein at a dose rate of 2.5 µg/kg. The mean molecular weight of the PVP was 360,000 with a free iodine concentration of less than 2 per cent and a specific activity of 40 µCi/mg. Blood samples (7ml) were collected into heparinised vacutainer tubes (Becton-Dickinson; Oxford) from the contralateral jugular vein after 8, 24, 30, 48 and 56 h. The

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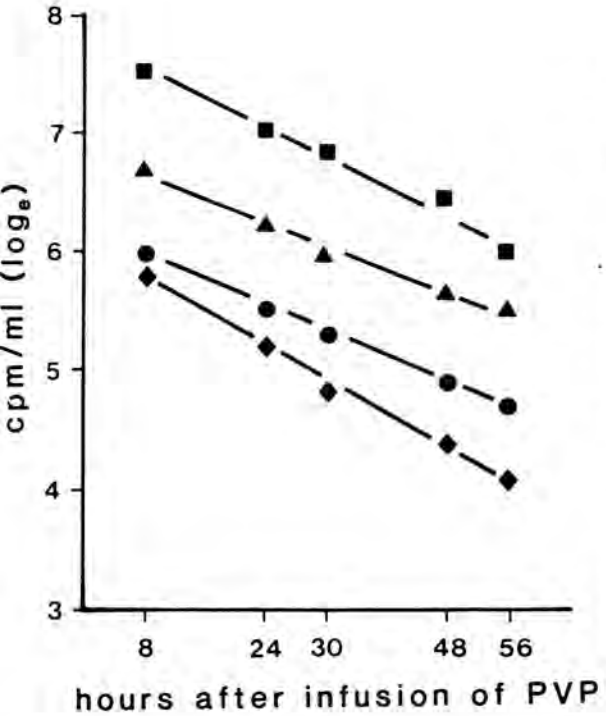
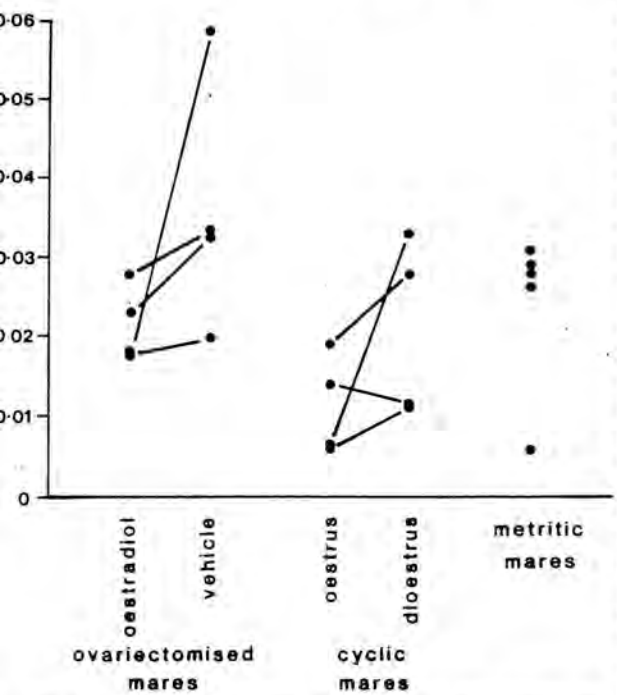


. Blood radioactivity of four mares after intravenous injection of 2.5 g of ^{125}I-labelled PVP

activity of 1 ml of whole blood was counted in a gamma ter (1282 Compugamma; LKB, Finland).

Rate of PVP clearance was calculated from the slope of the ession line of blood levels ($\log_e$) against time.



2. The effect of ovarian steroids and persistent endometritis on ance rates of ^{125}I-labelled PVP (expressed as the slope of the ession line of $\log_e$ against time)

Rates of clearance between genitally-normal and mares with persistent endometritis were compared using a *t* test.

Effect of circulating hormones were compared within mares by a paired *t* test. Results were considered significant when $P<0.05$.

Results

Fig 1 shows clearance of PVP from the blood of four representative mares. Administration of oestradiol to ovariectomised mares tended to decrease clearance rates compared with measurements taken before hormone treatment (Fig 2). Rates of clearance from three of the mares sampled during oestrus were less than during dioestrus. However, these differences were not statistically significant.

PVP clearance in mares with persistent endometritis was not significantly slower than that measured in genitally-normal mares.

Discussion

Clearance of PVP from horse blood eight hours after intravenous injection followed a pattern similar to that previously reported using mice (Morgan and Soothill 1975a) in which there was an initial rapid loss of low molecular weight PVP via glomerular filtration which was apparently complete by 8 h after injection. After 8 h, the half-life of PVP in mice was approximately 24 h which corresponds to that found in the mares in the present study.

Mares with persistent endometritis did not show any defect in macrophage function as measured by PVP clearance. Futhermore, elevated plasma concentrations of oestradiol in genitally-normal mares were not associated with increased PVP clearance. Rather, a trend towards decreased clearance was found. A recent study performed in male cats demonstrated either no change or a decrease in PVP clearance after administration of physiological levels of oestrogen compared with untreated controls (M. E. Stoddart, unpublished date). These results are in direct contrast to previous reports in which carbon clearance was increased in cycling mice and rats when plasma concentrations of oestrogen were elevated (Nicol, Vernon-Roberts and Quantock 1965) leading to the widely accepted hypothesis that oestrogen has a stimulatory effect on macrophages. However, it is interesting to note that at the lowest dosage of hormone administered to mice (0.001 mg), progesterone was in fact more stimulatory to macrophage activity than oestradiol, whereas at higher doses, which were pharmacological rather than physiological, the stimulatory effect of oestradiol became more apparent (Nicol *et al* 1965).

Both domestic and laboratory species are known to be resistant to uterine infection during periods of elevated circulating concentrations of oestrogen but highly susceptible during circulating concentrations of plasma progesterone (Rowson, Lamming and Fry 1953; Hawk 1959; Ganjam *et al* 1982). In guinea-pigs, ovarian oestrogen appeared to control an influx of macrophages into the endometrium (Nicol 1932). Furthermore, in women, the expression and staining of Ia molecules, which are involved in antigen recognition (Unanue *et al* 1984) was increased during periods of elevated plasma oestrogen (Tabibzadeh, Bettica and Gerber 1986). In mice, the rate of PVP clearance is directly related to affinity of antibody produced following parental immunisation (Morgan and Soothill 1975b). The liver and spleen are the major sites of clearance of PVP (Morgan and Soothill 1975a) and thus it is possible that this test is not a good indicator of local cellular activity in, for example, the reproductive tract. Since local application of antigen has been shown to be important in stimulating antibody production in the tract (Widders, Stokes, David and Bourne 1985), it is important

that the capacity of cells within the endometrium to present antigen is investigated. The PVP clearance test may prove to be useful in investigating other diseases in the horse where macrophage dysfunction is suspected.

Acknowledgements

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Short Communications

Endometrial macrophage populations in genitally normal mares at oestrus and dioestrus and in mares susceptible to endometritis

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Keywords: horse; uterus; macrophage

Introduction

Persistent endometritis is an important cause of subfertility in mares. Both uterine contractility (Troedsson *et al.* 1993) and local immune defence mechanisms (Watson 1988) are thought to be involved in combating infection. Identification of the components of the immune cell population within the equine endometrium and the changes which occur in density and distribution of these cells in response to cycle stage and antigen challenge, is needed to understand the immune mechanisms responsible for elimination of endometritis.

Macrophages play a key role in elimination of infection both as phagocytes and in antigen presentation and have already been detected in the endometria of other species including women (Kamat and Isaacson 1987; Bulmer *et al.* 1991), mice (De and Wood 1990) and cows (Cobb and Watson 1995). However, occurrence and distribution of these cells has not been studied in the endometrium of the nonpregnant mare. In the present study we documented the distribution and number of endometrial macrophages during the oestrous cycle of genitally normal mares and in mares susceptible to persistent endometritis.

Materials and methods

Endometrial biopsies from 18 horse and pony mares, age 3–20 years, were collected according to the procedure described by Kenney (1978). Stage of cycle was determined by teasing with a stallion and confirmed by examination of the ovaries and uterus by transrectal ultrasonography. Six mares were in oestrus, 6 in dioestrus and 6 had been presented with a history of subfertility and persistent acute endometritis at the time of biopsy collection. The latter mares were in oestrus, had bacterial uterine infections of beta haemolytic streptococci with luminal fluid visible on ultrasonography and had not undergone any treatment for infection in the cycle prior to biopsy.

Biopsy samples were placed in OCT compound[1], snap frozen in an isopentane/dry ice slurry and then kept at -70°C until further processing. Cryostat sections 6–8 μm thick were cut from each biopsy sample and placed on biobond-coated[2] slides. One section from each biopsy sample was stained with haematoxylin and eosin (H&E) to ensure that the morphology of the tissue sample had been adequately preserved. The rest of the sections were stained by an avidin-biotin complex staining method, using primary monoclonal antibodies (WS 33 and WS 36) as monocyte/macrophage markers. The specificities of these monoclonals have been described by Kydd *et al.* (1994). Both WS 33 and WS 36 label monocyte/macrophage-like cells, although WS 33 is thought to label a subpopulation of granulocytes as well. The avidin-biotin peroxidase kits were purchased from Vector Laboratories[3]. The staining method used has been described previously (Watson and Thomson 1996).

Endometrial sections were included in which the primary monoclonal antibody was excluded, to detect endogenous peroxidase activity or nonspecific binding by the biotinylated antibody. Cryostat sections of equine lymph nodes were also included on each slide to act as positive controls.

Quantification

The number of positively staining cells in 5 randomly selected microscope fields (using the x10 objective) was counted for each section, to estimate the size of the macrophage population in the uterine *lamina propria.* The mean of these 5 values was calculated for each group of mares for statistical analysis.

Statistical analysis

A one way ANOVA, with least significance difference mean comparisons, where appropriate, was performed to evaluate the effects of cycle stage on endometrial macrophage populations in genitally normal mares at oestrus and at dioestrus, and of acute persistent bacterial infection in susceptible mares. A paired *t* test was used to compare the number of cells staining positively with each monoclonal antibody.

Results

Endometrial staining

For each monoclonal antibody, positively-stained cells were found scattered throughout both *stratum spongiosum* and *stratum compactum* in all mares (Figs 1 and 2). These cells were present

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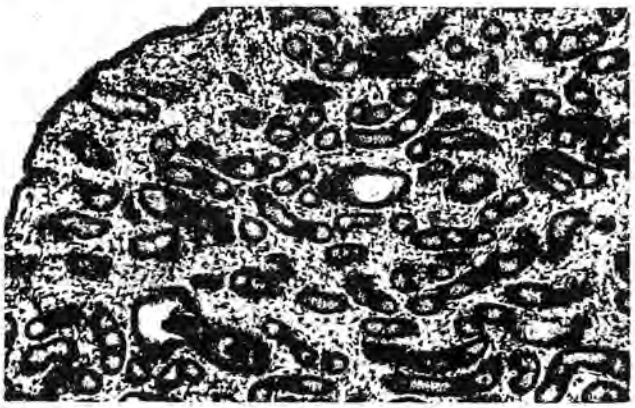

Fig 1: Endometrial biopsy from a mare in oestrus stained by immunohistological methods using WS33 antibody (x10).

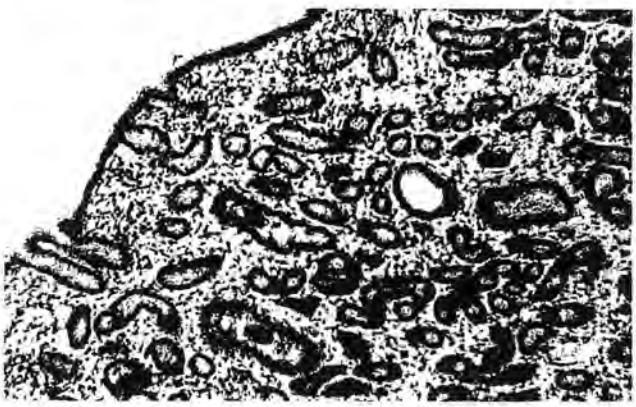

Fig 2: Endometrial biopsy from a mare in oestrus stained by immunohistological methods using WS36 antibody (x10).

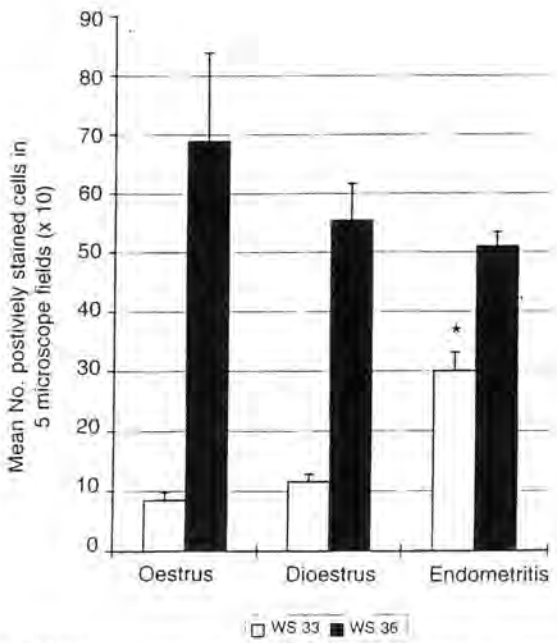

Fig 3: Mean (± s.e.) number of endometrial cells staining positively with each monoclonal antibody at oestrus and dioestrus in genitally normal mares and in oestrous mares with endometritis. P<0.001 for WS33-stained cells in mares with endometritis compared with genitally normal mares in oestrus and dioestrus.

Fig 4: Endometrial biopsy from mare with endometritis stained by immunohistological methods using WS33 antibody for macrophages and neutrophils. Note subepithelial accumulation of positively staining cells (x10).

Fig 5: Section of equine lymph node stained using antibody WS33. Positively staining cells are distributed throughout the lymph node (x4).

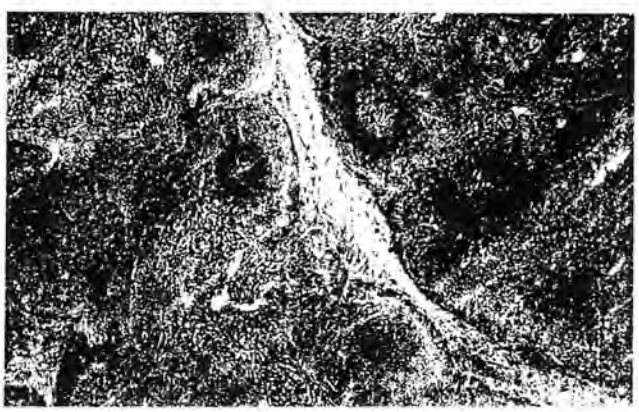

Fig 6: Section of equine lymph node stained using antibody WS36. Positively staining cells are located predominantly within the medullary cords and sinuses (x4).

in greater density in the *stratum spongiosum* than in the *stratum compactum*. Positively stained cells were often located in periglandular accumulations in the *stratum spongiosum*, but were absent from both luminal and glandular epithelium.

WS 33

There was no significant difference in the numbers of positively stained cells in the endometrium of genitally normal mares at oestrus and dioestrus (Fig 3). The increase in number of positively staining cells in mares with endometritis compared with genitally normal mares at both oestrus and dioestrus was

highly significant (P<0.001). The majority of stained cells in mares with endometritis were situated in the sub-epithelial region of the *stratum compactum* (Fig 4). Serial sections (H&E) revealed that most of these subepithelial cells were neutrophils.

WS 36

No significant difference occurred between the numbers of positively stained cells in the endometrial samples from mares in oestrus, dioestrus or with endometritis (Fig 3). Significantly more cells stained positively using the WS36 antibody than the WS33 antibody (P<0.001). Sequential sections stained with H&E showed that cells staining with WS36 had a macrophage/monocyte-like morphology.

Lymph node staining

Lymph node samples showed cell staining in the following locations: WS 33 scattered throughout the entire lymph node (Fig 5), whereas WS 36 were situated predominantly within the lymph node medullary sinuses (Fig 6). Samples in which the primary antibody was excluded did not show any positive staining.

The quality of staining was better using WS 36, which produced a clear and well defined staining pattern (Fig 2). WS 33 produced a diffuse staining pattern which was less easy to interpret, especially when 2 or 3 cells were closely apposed (Fig 1).

Discussion

From our results it appears that, in the mare, stage of cycle does not influence the number of macrophage-like cells in the endometrial *lamina propria*. Similarly, in genitally normal mares, neither T nor B lymphocytes appear to be affected by stage of cycle (Watson and Dixon 1993; Watson and Thomson 1996). Studies of endometrial macrophage populations in other species have produced varied results. In the mouse (De and Wood 1990), oestrogen and progesterone stimulate the murine endometrium to produce macrophage colony-stimulating factor (M-CSF) which is responsible for monocyte migration, maturation and differentiation. Fluctuations of these hormones were therefore proposed to cause the variations observed in macrophage populations with cycle stage in this species. However, in the endometrium of women and cows (Starkey *et al.* 1991; Cobb and Watson 1995), macrophage numbers did not change with cycle stage, although Bulmer *et al.* (1991) reported an increase premenstrually in women.

Cobb and Watson (1995) found that, in the cow, more macrophages were generally present within the *stratum spongiosum* compared with the *stratum compactum* and this was also the case in the mare. The absence of macrophages in the luminal and glandular epithelia in the cow agrees with the findings in the mare. However, in this present study, periglandular accumulations of macrophage-like cells were frequently seen in the *stratum spongiosum* but were reported to be rare in that of the cow.

It seems surprising that the samples from mares with endometritis did not show a significant increase in positively stained cells in the endometrium using the WS 36 monoclonal. The significant increase in cells marked with WS 33 in these susceptible mares was due to an increase in neutrophil numbers, as WS 33 also labels a subpopulation of granulocytes (Kydd *et al.* 1994). An influx of neutrophils is expected at sites of tissue inflammation and bacterial invasion. Similarly, an influx of macrophages could be expected, although this did not appear to be the case in our study. B cells and T cells (of which CD4+ cells are more numerous than CD8+ cells) increase in number in the endometrium of mares with endometritis (Watson and Thomson 1996). Perhaps the failure of macrophage numbers to increase in line with the increase in CD4+ helper T cells results in an inadequate amount of phagocytosis an/or antigen presentation, which could be a possible contributing factor to the susceptibility to infection in these mares. However, in other species, CD4+ cells are heterogeneic with respect to cytokine production. So although the cells were identified as CD4+, we have no information in the horse on specific cytokines produced and therefore their function.

Further study of the type and function of macrophages in the equine endometrium, requires that the identity of macrophage surface receptors which the WS 33 and WS 36 monoclonals are recognising are determined. Furthermore it has been reported in laboratory animals that monocytes/macrophages/dendritic cells may transdifferentiate into one another under appropriate conditions (Goerdt *et al.* 1996). It may be that these 2 monoclonals recognise surface markers common to phenotypically and functionally different macrophage sub-populations. If so, it is possible that proportional changes, as opposed to variations in the macrophage population as a whole, were present in the endometrial samples in the present study. This should be clarified once further information on phenotype and function of equine macrophage populations becomes available.

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Manufacturers' addresses

[1]Miles Inc., Elkhart, Indiana, USA.
[2]British BioCell International, Cardiff, UK.
[3]Peterborough, Cambs, UK.

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Reviewed

THE EFFECTS OF DEXAMETHASONE SODIUM PHOSPHATE ON MARES WITH EXPERIMENTALLY-INDUCED ENDOMETRITIS

A. M. McDonnell[1] and E. D. Watson[2]

SUMMARY

Mares with experimentally-induced endometritis were treated systemically with a therapeutic dose of 40 μg/kg bodyweight of dexamethasone sodium phosphate by intravenous injection for three consecutive days in the presence or absence of intrauterine antibiotic treatment. The cytologic, microbiologic, ultrasonographic and histologic responses of the uterus to treatments were monitored. The dexamethasone-only treated mares showed a greater inflammatory response to infection than any of the other treatment groups, especially as judged by endometrial histology where there was widespread neutrophil and plasma cell infiltration of the stratum compactum and stratum spongiosum. Mares treated with antibiotic or antibiotic plus dexamethasone showed only a mild degree of endometrial inflammation. This study has demonstrated that, at the dose level used in this experiment, dexamethasone sodium phosphate did not appear to have any beneficial therapeutic effects in reducing the inflammation associated with endometritis compared to treating with antibiotic alone.

INTRODUCTION

Approximately 25% of mare subfertility has been attributed to bacterial endometritis.[1] Reduction in the conception rates of mares with endometritis is probably due to the presence of neutrophils in the uterine lumen which are cytotoxic for gametes and embryos.[2]

The major sources of bacterial contamination of the uterus are during coitus, parturition and veterinary gynecological examination procedures which involve entering the uterus. Various treatment regimens have been adopted in an attempt to cure endometritis and prevent chronic inflammation. It has been reported, anecdotally, that systemic treatment with dexamethasone sodium phosphate has been beneficial in reducing the adverse inflammatory reaction associated with endometritis. In the present study, the effect of dexamethasone sodium phosphate in mares with experimentally-induced endometritis was investigated. We also examined the effect of dexamethasone on the progesterone-dominated uterus in the absence of bacterial infection.

MATERIALS AND METHODS

Experimental design

Six pony mares were selected on the basis of minimal evidence of uterine inflammation on histological examinations of endometrial biopsy samples (Grade I or IIA[3]) and no bacterial growth from swabbings of the endometrium. The mares were randomly assigned to receive one of four treatments. On successive occasions treatments were switched so that by the end of the project most mares had received all treatments.

The protocol for the project was as outlined in Table 1. Mares were allowed one week's rest period between experiments. Mares usually clear any residual uterine infection

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Table 1. Experimental design.

Day 0	Start progesterone therapy; 150mg daily i.m.
Day 5	Per rectum examination of the uterus by palpation and ultrasonography. Culture of endometrial swabbings. Uterine lavage. Infuse bacteria.
Day 6	Ultrasonographic examination of the uterus.
Day 10	Per rectum examination of the uterus by palpation and ultrasonography. Culture of endometrial swabbings. Start daily treatments.
Day 12	Per rectum examination of the uterus by palpation and ultrasonography. Uterine lavage. Culture of endometrial swabbings. Last treatment.
Day 16	End progesterone treatment. Endometrial biopsy.
Day 17	Prostaglandin $F_{2\alpha}$ analogue i.m.

spontaneously once progesterone treatment has ceased, but any mare which remained infected was treated appropriately.

Endometritis was evaluated by manual and ultrasonographic examination per rectum, culture of endometrial swabbings, endometrial cytology and endometrial biopsy. Manual palpation per rectum was performed to assess uterine and cervical tone and uterine enlargement. Per-rectum ultrasonographic examination of the uterus was used to obtain accurate measurement of uterine size and to determine if fluid was present.

Procedures which involved entering the uterus were carried out as aseptically as possible. The tail was wrapped in a plastic sleeve and elevated away from the perineal region. After emptying the rectum, the perineal region was scrubbed three times with providone-iodine scrub and rinsed thoroughly between each wash. The sampling procedures were carried out using a clean plastic rectal sleeve over which a sterile surgeon's glove was worn, and the hand was lubricated using sterile water soluble lubricant.

Induction of endometritis

To induce susceptibility to endometritis, each mare was injected intramuscularly daily with 150 mg of progesterone in cottonseed oil. This treatment regimen has been shown to induce changes in plasma hormones and in the histological morphology of the uterus consistent with diestrus.[4,5] Treatment with progesterone continued for 16 consecutive days.

Preparation of bacteria for intrauterine infusion

Streptococcus zooepidemicus was isolated from the uterus of a mare with acute endometritis and the bacteria were stored at -70°C in brain heart infusion broth containing 10% glycerol. On the day before infusion, an aliquot was thawed and inoculated into brain heart infusion broth and incubated overnight at 37°C. Bacteria were prepared for infusion by washing twice in sterile phosphate buffered saline (PBS, pH 7.2), and resuspending them at $1x10^9$/ml by calibration using a spectrophotometer. The organisms were placed in 20ml sterile PBS and infused into the uterus using a sterile infusion pipette. The endometrium of all mares was swabbed prior to bacterial infusion to ensure that no pre-existing bacterial infection was present and also 5 days after bacterial infusion to ensure that endometritis had been established.

Table 2. Ultrasonographic examinations of the uterus of mares on the last day of treatment, prior to administration of the last treatment. Measurements were recorded using 5mHz linear probe.

Mare	Treatment			
	Water	Ampicillin	Ampicillin+ Dexamethasone	Dexamethasone
1	+	NF	+	20 mm
2	27 mm	-	-	22 mm
3	6 mm	27 mm	NF	-
4	NF	NF	17 mm	24 mm
5	35 mm	NF	17 mm	24 mm
6	6 mm	NF	NF	-

NF = no fluid.
+ = small amount of fluid visible but not discrete within the uterine lumen.
- = mare not included in this experiment.
All measurements made consistently at the same locations, ie widest diameter of the uterine body. All measurements recorded as millimeters of fluid in the uterine body.

Preparation of uterine flushings

Uterine flushings were collected on days 5 and 12 of progesterone treatment. The samples were centrifuged at 1000g for 10 min at 4°C and the cell deposit was resuspended in 1 ml PBS. The cells were counted using a hemocytometer and assessed for viability by exclusion of Trypan blue dye. Differential cell counts were performed on a smear prepared using a cytocentrifuge. The preparations were stained using Leishman's stain and 200 cells were counted.

Treatments

Mares received the following treatments on three consecutive days.

(A) 2 g of ampicillin as sodium salt[a] intrauterine in 50 ml of sterile water.

(B) 50 ml of sterile water BP[b] for injection, pH 6.9, intrauterine.

(C) 2 g of ampicillin in 50 ml of sterile water intrauterine and 40 μg/kg of dexamethasone sodium phosphate[c] intravenously.

(D) 40 μg/kg bodyweight of dexamethasone sodium phosphate intravenously.

Two mares out with the experimental group were given daily intramuscular injections of progesterone in cottonseed oil (150 mg) over the same period of time as the main experimental group but were not infused with the bacteria on day 5. These mares received 40 mg/kg bodyweight dexam-

[a]Penbritin Veterinary Injectable, Smithkline Beecham Animal Health Ltd, Tadworth, England.
[b]BK Veterinary Products Ltd, Bury St Edmonds, England.
[c]Dexadreson, Intervet UK Ltd, Cambridge, England.

Table 3. Cytological examination of uterine lavage fluid collected before infusion of bacteria and on the last day of treatment.

Mare	Treatment							
	Water		Ampicillin +Dexamethasone		Ampicillin		Dexamethasone	
	pre	post	pre	post	pre	post	pre	post
1	<5%N	92%N	10%N	100%N	5%N	92%N	<5%N	100%N
2	<5%N	71%N	-	-	-	-	<5%N	100%N
3	3%N	99%N	5%N	72%N	5%N	70%N	-	-
4	5%N	99%N	19%N	65%N	15%N	100%N	<5%N	97%N
5	<5%N	100%N	6%N	92%N	10%N	61%N	<5%N	63%N
6	8%N	100%N	5%N	92%N	10%N	61%N	-	-

N = Neutrophils
- = mare excluded from the experiment.

Table 4. Hemocytometer counts of cells in uterine lavage fluid collected prior to bacterial infusion and on the last day of treatment ($x10^5$/ml)

Mare	Treatment							
	Water		Ampicillin +Dexamethasone		Ampicillin		Dexamethasone	
	pre	post	pre	post	pre	post	pre	post
1	32	187 (80%)	40	54 (68%)	29	124 (70%)	18	334 (55%)
2	34	126 (85%)	-	-	-	-	20	188 (50%)
3	16	135 (87%)	15	117 (65%)	17	45 (62%)	-	-
4	19	39 (85%)	30	45 (56%)	34	56 (55%)	21	363 (11%)
5	15	36 (75%)	15	23 (60%)	15	30 (61%)	10	162 (44%)
6	27	40 (88%)	20	57 (70%)	27	85 (84%)	-	-

Figures in parentheses are percentage viable neutrophils as determined by exclusion of Trypan blue dye.
Pre = pre bacterial infusion.
Post = last day of treatment, prior to infusion of last treatment.

ethasone sodium phosphate on day 10 to 12, inclusive, and endometrial biopsy samples were collected on day 16.

All biopsy specimens were examined without prior knowledge of their origin. Inflammatory responses were classified as mild, moderate, marked or severe depending on the degree of inflammation and neutrophil infiltration into the stroma, degree of margination of neutrophils in endometrial blood vessels, presence of inspissated secretion and neutrophils in endometrial glands, presence of edema in the stratum compactum and transepithelial migration of neutrophils.

Microbiology

Endometrial swabbings were collected aseptically on day 5, prior to bacterial infusion, day 10 and on day 12 using double guarded endometrial swabs,[d] which were rotated against the endometrium for 10 sec. All swabs collected were plated onto horse blood agar within three hours of collection and incubated at 37°C for 48 h. Plates were graded by recording 0 to 20 colonies as 1+, greater than 20 colonies as 2+, 3+ or 4+ depending on the extent of bacterial growth.

[d]Equivet, Dunwood, Bridge of Don, UK.

Ultrasonic evaluation of the reproductive tract

The greatest diameter of fluid present in the body of the uterus was recorded at each examination. Often, this was not palpable on rectal examination.

RESULTS

Ultrasonographic findings

Mares scanned one day after infusion of bacteria showed no evidence of fluid in the uterus. At the end of treatment results ranged from 6 mm to 35 mm in all the groups (Table 2). The dexamethasone-only treated group showed the most consistent fluid diameters in the uterine body, ranging from 20 to 24 mm post treatment. This fluid was hypoechoic with echogenic particles indicative of a purulent secretion.

Uterine cytology

Before infusion of bacteria percentage of neutrophils was less than 20% whereas after treatment neutrophils comprised 61% to 100% of uterine cells (Table 3). There appeared to be

ble 5. Post-treatment microbiological culture results of dometrial swabbings collected on day 12.

re	Treatment			
	Water	Ampicillin	Ampicillin+ Dexamethasone	Dexamethasone
	4+	3+	2+	4+
	4+	-	-	4+
	2+	2+	1+	-
	4+	2+	1+	4+
	3+	NG	3+	4+
	1+	2+	1+	-

Mare excluded from that experiment.
= No bacterial growth.

Table 6. Histological evaluation of endometrial biopsies collected 4 days after the last treatment.

Mare	Treatment			
	Water	Ampicillin	Ampicillin+ Dexamethasone	Dexamethasone
1	moderate	mild	mild	severe
2	moderate	-	-	severe
3	marked	mild	mild	-
4	marked	mild	mild	severe
5	marked	mild	mild	severe
6	marked	moderate	mild	-

All biopsies were read without prior knowledge of their source of origin.

considerable amount of variation between horses in the sponse to treatments. Compared with the other treatment oups, cytology from the dexamethasone-treated group con-stently showed more degenerate and ghost neutrophils and sophilic and degenerate cells with nuclear remnants, con-ning phagocytosed bacteria. There were fewer viable neu-ophils in this group compared to the other treatment groups able 4).

emocytometer counts

Hemocytometer counts are shown in Table 4. Counts nged from $15x10^5$/ml before infusion of bacteria to $3x10^5$/ml post-treatment. In the dexamethasone-treated imals, there was a consistently large difference between e- and post-treatment compared to the other groups. The mpicillin-treated groups had cell numbers post-treatment at were not dissimilar from the water-treated group, but nically, on ultrasonographic examination and on quantita-e microbiological scoring, they had less inflammatory udate and fewer bacteria, respectively, compared to the ater-only group.

crobiology

Mares swabbed prior to bacterial infusion on day 5 of ogesterone treatment were found to have no bacterial growth. dometrial swabbings from all mares had heavy (4+) bacterial owth prior to treatment. The water-only treated animals main-ned a uterine infection throughout the period of treatment able 5). The ampicillin-only and ampicillin + dexamethasone-ated mares showed a quantitative decrease in bacterial owth following treatment. The bacterial growth in the dexam-asone-only treated group remained at pre-treatment levels.

dometrial biopsy

Mares which received water-only treatment had a mod-te to marked inflammatory response as indicated by mar-ation of neutrophils in endometrial blood vessels (Table 6). ere was a moderate, scattered neutrophil infiltration into stratum compactum and stratum spongiosum and moder-amounts of inspissated eosinophilic debris in endometrial nds. There was also a moderate amount of transepithelial gration of neutrophils. The lumen of the uterus also con-tained a moderate amount of eosinophilic debris containing neutrophils.

The ampicillin-treated mares had a generally mild histological response to treatment i.e., a scant amount of eosinophilic debris in the uterine lumen, and scattered infrequent neutrophil infiltration into the stratum compactum. There was scattered, infrequent gland distension with eosinophilic debris within the glands and moderate neutrophil margination in blood vessels. The ampicillin + dexamethasone-treated mares had a mild histologic response also, similar to the ampicillin-treated group.

The histological response to dexamethasone treatment was uniformly severe, and characterized by an intense neutrophil infiltration of the stratum compactum and stratum spongiosum. Two of the mares had widespread plasma cell infiltration of the stratum compactum and stratum spongiosum and the other two had moderately frequent plasma cell infiltration of the stratum compactum and stratum spongiosum. There was distension of the endometrial glands with eosinophilic secretion containing neutrophils and marked margination of neutrophils in endometrial blood vessels. The lumen of the uterus contained neutrophils and there was widespread transepithelial migration of neutrophils.

The two mares which received dexamethasone treatment in the absence of any bacterial infection showed no histological evidence of uterine inflammation.

DISCUSSION

In the present study, the effect of therapeutic doses of dexamethasone in the presence or absence of appropriate antibiotic therapy was investigated using the experimental model for endometritis described by Colbern et al[4] and Watson.[5] The progesterone-dominated uterus is highly susceptible to the establishment of infection by invading microorganisms, because of reduced neutrophil function,[6,7] reduced opsonizing capacity of specific antibodies[8] and a reduced ability to physically clear uterine infection.[9] Under estrogen influence, uterine pathogens are rapidly cleared from the uterus (Evans et al, 1986). Use of the progesterone-treatment model created a uniform population to study the

effect of dexamethasone and other treatment regimens.

We have shown that treatment of mares with acute endometritis with dexamethasone resulted in a greater inflammatory response than treatment with ampicillin alone or a combined treatment of ampicillin and dexamethasone. On ultrasonographic examination, the dexamethasone-treated group showed the most consistent increase in inflammatory exudate in the uterus compared to the other groups. It is possible that some of the fluid visible in the uterine lumen post-treatment may have been the infused treatment, as the cervix under the influence of progesterone should be tightly closed. However, some of these mares had a vulvar discharge, indicating that fluid was in fact draining to the exterior.

Total cell counts from uterine lavage fluid collected before infusion of bacteria and post-treatment showed considerable within- and between-horse variation in response to bacterial infection. However, the dexamethasone-treated mares showed the most consistent increase in cell counts. The histological response to infection in the dexamethasone-treated group was more severe than the other treatment groups. There was an intense invasion of the endometrium by neutrophils and plasma cells. The ampicillin-only and ampicillin + dexamethasone-treated mares did not differ greatly in their histological response to inflammation. The two mares treated with dexamethasone in the absence of uterine intervention showed no alteration in endometrial histology. Therefore, dexamethasone by itself did not act to cause uterine inflammation.

Dexamethasone is a known systemic immunosuppressant,[10] but in the present study, examination of endometrial biopsies showed that dexamethasone did not appear to inhibit the ability of neutrophils to gain access to inflammatory sites. In the ampicillin plus dexamethasone-treated groups there was a quantitative decrease in bacterial growth between pre- and post-treatment uterine cultures. However in the dexamethasone-only treated mares, bacterial growth did not alter during treatment. Ampicillin sodium is widely used for treatment of endometritis[11] in spite of forming a precipitate on the endometrium.[12] In the ampicillin-treated group, mixed bacterial growth was present on the last day of treatment. The lack of response to antibiotic treatment was shown to be due to resistance of the organisms present to ampicillin, as determined by sensitivity tests. These organisms, staphylococci and *E. coli*, are normal commensals of the anterior vagina and were probably inadvertently introduced during treatment infusion[13] and reflect the problems encountered when these manipulations are performed in mares under the influence of progesterone.[13,14]

The findings of the present study contrast with those of Colbern et al[4] who administered 40 mg of dexamethasone sodium phosphate i.m. daily for 16 consecutive days with daily i.m. injections of progesterone in oil. They reported that these mares had a less dramatic inflammatory response to inoculation of bacteria than mares receiving progesterone alone. However, 14 days later the dexamethasone-treated mares had more severe inflammatory responses in endometrial biopsy samples. Reasons for this treatment strategy, or for results obtained were not given.

In conclusion, it would appear that dexamethasone alone or in combination with antibiotic did not appear to have any beneficial therapeutic effect in resolving the inflammation associated with acute endometritis.

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Soluble oestrogen and progesterone receptors in the endometrium of the mare

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Summary. Receptors for progesterone and oestrogen were measured in cytosol extracted from the endometrium of mares. Samples of endometrium were obtained from mares during the oestrous cycle and during other physiological and pathological states, and from mares with persistent endometritis. The concentration of binding sites was high around oestrus and early in dioestrus and was low by mid- to late dioestrus. Numbers of receptors were low in mares sampled on Day 15 of pregnancy and in mares in prolonged dioestrus, whereas receptor concentrations in ovariectomized mares were high. In 2 mares with endometritis, receptor concentrations were low in early dioestrus, which was markedly different from mares with a normal reproductive tract. The results of this study indicate that ovarian steroids may play a role in regulating concentrations of soluble endometrial receptors in the mare.

Introduction

The uterine environment is important in establishing and maintaining pregnancy in the mare. The uterus is a hormone-responsive organ. The function and morphology of the endometrium are controlled by oestrogen during oestrus and by progesterone during dioestrus or pregnancy. High concentrations of oestrogen and progesterone are sequestered in the endometrium from the blood because of the presence of specific hormonal receptors within the endometrium.

There is no information on concentrations of steroid hormone receptors in the endometrium of the mare. In other species, concentrations of endometrial receptors vary throughout the oestrous cycle, and the variations control steroid hormone action on the uterus. When circulating concentrations of oestrogen are high, i.e. during prooestrus and oestrus, concentrations of endometrial receptors for progesterone and oestrogen tend to be at their highest in the cow (Senior, 1975; Zelinski *et al.*, 1982), ewe (Koligian & Stormshak, 1977; Miller *et al.*, 1977), and bitch (Fernandes *et al.*, 1989). During dioestrus, when circulating concentrations of progesterone are high, concentrations of endometrial oestrogen and progesterone receptors tend to decrease in the cow (Zelinski *et al.*, 1982; Vesanen *et al.*, 1988) and ewe (Koligian & Stormshak, 1977; Miller *et al.*, 1977). However, in swine, oestrogen receptor concentrations peak during mid-dioestrus (Deaver & Guthrie, 1980).

The present study was performed to measure changes in concentrations of cytoplasmic receptors for progesterone and oestrogen in the mare endometrium during the oestrous cycle and under various physiological and pathological conditions.

Materials and Methods

Fifteen Standardbred, Thoroughbred and Quarter Horse mares weighing 450–550 kg were used. An endometrial biopsy was collected from each mare, examined histologically, and categorized using the criteria of Kenney & Doig (1986). Thirteen of the mares had a Category I or IIA endometrium, and 2 of the mares had chronic endometritis with a Category III endometrium. All mares were teased daily with a stallion for signs of oestrus. After oestrus was detected in mares, ovarian follicular development was monitored daily by ultrasonography until ovulation. Using the technique described by Kenney

(1978), endometrial biopsy samples (n = 4) were collected from the base of both uterine horns of each mare on Day 3 of oestrus, day of ovulation (Day 0) and Days 3, 6, 10 and 15 of dioestrus over 3 cycles. Biopsy samples were collected on two different days during any one cycle, i.e. Day 3 of oestrus plus Day 10 of dioestrus, Day 3 of dioestrus plus Day 15 of dioestrus and Day 0 plus Day 6 of dioestrus. On each occasion, an additional biopsy sample was placed in Bouin's solution for histological evaluation using the criteria of Kenney & Doig (1986). At a subsequent cycle, 6 mares were bred and endometrial biopsy samples were collected on Day 15 of pregnancy. Endometrial biopsy samples were collected from both mares with chronic endometritis on Day 3 of oestrus and Days 3 and 10 of dioestrus. Endometrial biopsy samples also were obtained on 1 occasion from 5 ovariectomized mares and from 2 mares in prolonged dioestrus after at least 20 days of constantly elevated progesterone concentrations in blood samples. Each time biopsy samples were obtained, blood samples (20 ml) were collected into evacuated heparinzed tubes. Plasma was frozen at -20°C until assayed for concentrations of progesterone.

Tissue extraction

Endometrial biopsy tissue was stored at -70°C until extraction. During extraction, the tissue was maintained at 0–4°C in an ice bath. The tissue was weighed, minced and placed in a ground glass homogenizer with 6 volumes (w:v) TE buffer (10 nM Tris HCl, 1·5 mM EDTA, pH 7·6) for oestradiol receptors and with 5 volumes TEDG buffer (10 mM Tris, 1mM disodium EDTA, 3mM dithiothrietol and 10% glycerol, pH 7·6) for progesterone receptors, with 10 mM fresh sodium molybdate added just before each assay. After homogenization, the tissue was centrifuged at 18 000 g for 30 min at 4°C. The supernatants (cytosols) were decanted and stored at -70°C until assayed (within one week). Cytosol protein concentrations were measured by the method of Lowry *et al.* (1951) using a BSA standard.

Oestrogen and progesterone receptor assays

Receptors were measured using a method similar to that described by Fernandes *et al.* (1989). Optimal assay conditions were determined by varying incubation time and temperature. Cytosol (200 µl; 1:6 oestradiol, 1:32 progesterone) was incubated with 100 µl 30 nM (2,4,6,7)-[^{3}H] oestradiol (103·8 Ci/mmol; New England Nuclear, Boston, Massachusetts) or [^{3}H]-progesterone (R502) (80 Ci/mmol; New England Nuclear) for 24 h at 4°C (total binding). The saturating concentration of [^{3}H] steroid was determined by preliminary saturation curve analysis using cytosol samples from several mares. To determine non-specific binding, duplicate samples of each cytosol were incubated in the presence of 100 ul of 100-fold excess unlabelled oestradiol or R502. After incubation overnight at 4°C, 800 µl dextran-coated charcoal (5% acid-washed charcoal, 0·5% dextran dissolved in TEDG buffer) was added to the tubes to remove unbound steroid. The tubes were incubated at 4°C for 10 min and then centrifuged at 3000 g for 15 min. The supernatant was

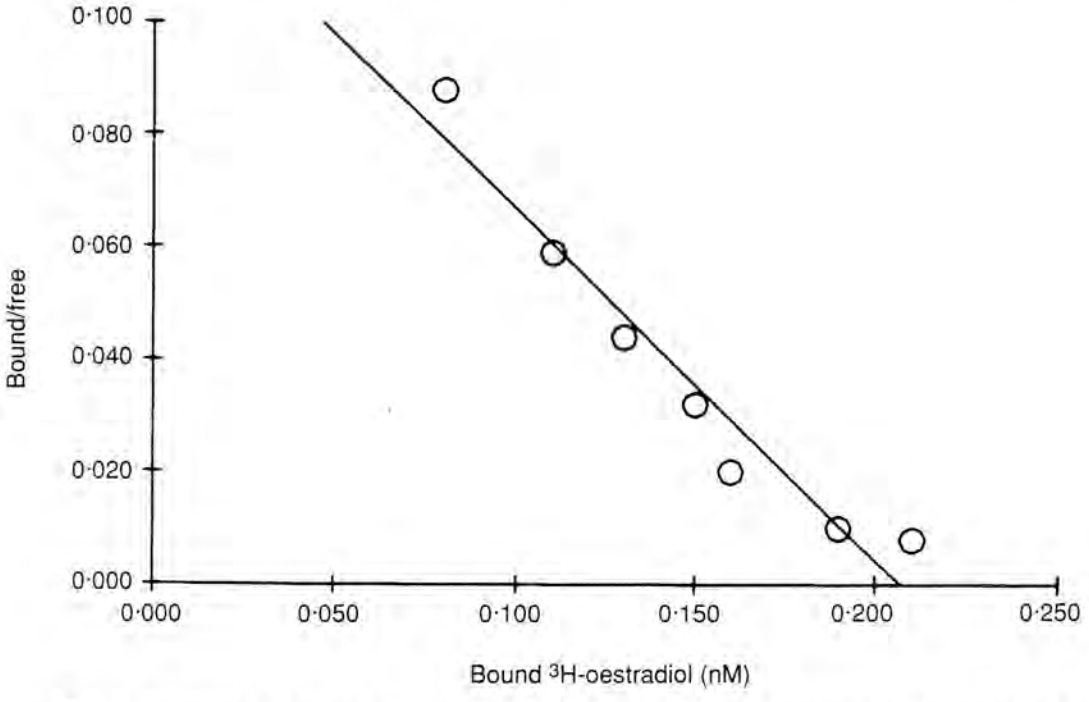

Fig. 1. Scatchard-plot analysis of specifically-bound labelled oestradiol in a pooled sample of equine endometrial cytosol.

decanted into scintillation vials and 10 ml scintillation fluid added (1 gallon toluene, 15 g PPO). Radioactivity was measured using a Beckman LS 3150T scintillation counter.

The moles of specifically bound steroid were calculated by subtracting the non-specific binding from total binding. Scatchard plots (Fig. 1) were constructed to calculate the binding data. Receptor concentrations were expressed as fmol/mg cytosol protein. Control samples were included in each assay to check inter-assay variation and to ensure that there were no losses in receptor binding during storage.

The nature of the receptor was determined by incubating cytosol with protease (20 μg/ng protein) for 6 h at 4°C prior to assay. The specificity of the assays was tested by competitive inhibition of [^{3}H] oestrogen or [^{3}H] progesterone binding by oestradiol, testosterone, progesterone and hydrocortisone. Tissue specificity of receptors was assessed using samples of muscle and spleen.

Plasma progesterone assay

Progesterone concentrations were used to confirm stage of cycle. Progesterone was assayed in plasma using the assay described by Watson and Hinrichs (1989). Reagents were obtained from Diagnostic Products Corporation, Los Angeles, California. Standard concentrations of progesterone were re-suspended in ovariectomized mare plasma. The antiserum had the following cross-reactivites: progesterone 100%, 11-deoxycorticosterone 1·7%, 11-deoxycortisol 2·4%, 20α - dihydroprogesterone 2%, 5β-pregnan-3,20-dione 1·3% and other steroids tested < 1%. The limit of detection of the assay was 25 pg/ml. The within- and between-assay coefficients of variation were 6 and 9%, respectively.

Statistical analysis

A one-way analysis of variance, with least significant difference mean comparisons where appropriate, was performed to evaluate the effects of day of oestrous cycle and physiological or pathological conditions on receptor concentrations.

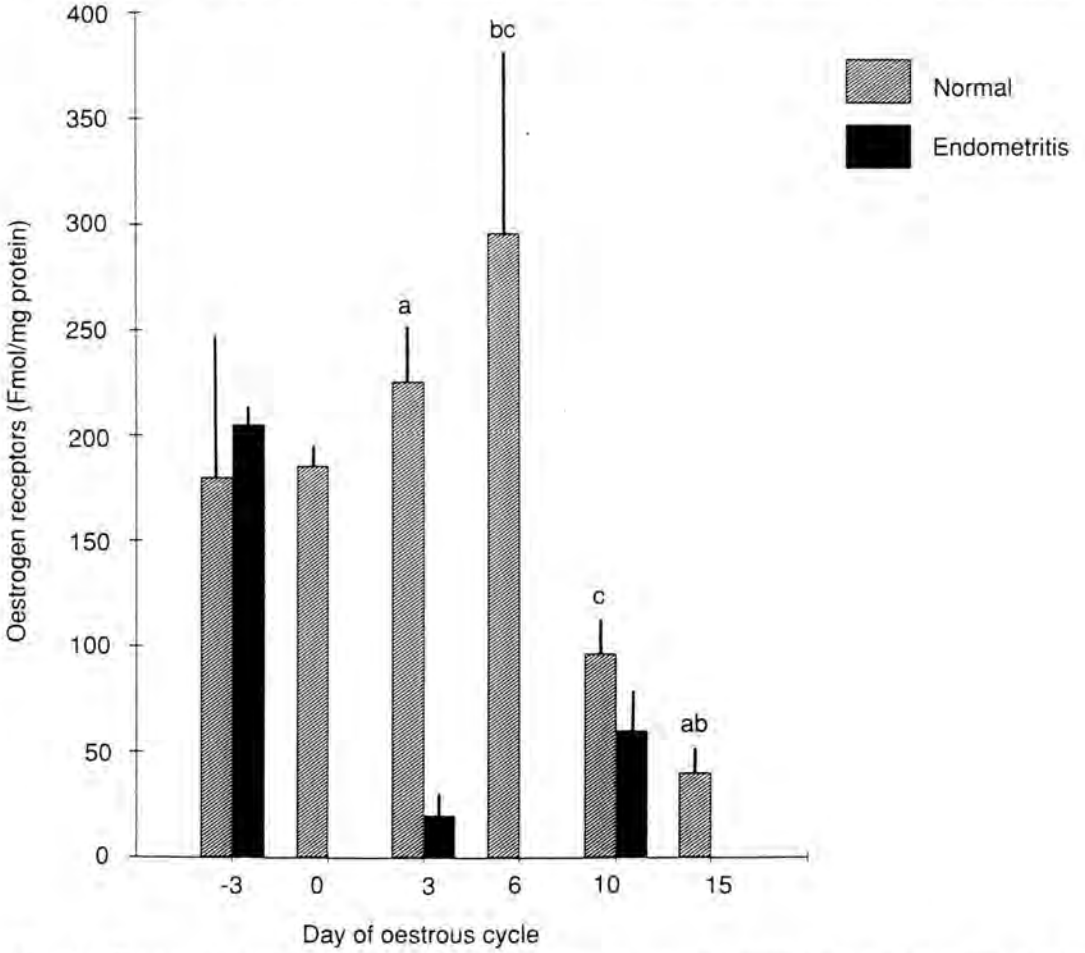

Fig. 2. Concentrations of oestrogen receptors in the endometrium of the mare during the oestrous cycle. n = 3–6 per group. Day -3 = Day 3 of oestrus. Columns with similar superscripts are significantly different, $^{a}P < 0{\cdot}01$, $^{b,c}P < 0{\cdot}05$.

Results

Receptor binding was optimal when a 24-h incubation period was used. Use of temperatures higher than 4°C resulted in a rapid drop in specific binding. Cross-reactivity of the receptor assays with other steroids tested was negligible. Specific oestrogen or progesterone binding was very low in muscle and spleen cytosols. Specific binding was lost after treating the cytosol with protease. This indicates the proteinaceous nature of the receptors.

Histological evaluation of endometrial biopsy samples showed that none of the mares had evidence of acute endometritis at the time of biopsy. Figure 1 shows a typical Scatchard plot of results obtained by incubating cytosol with increasing concentrations of labelled oestradiol. Concentrations of oestrogen receptors were high during oestrus and early dioestrus, with maximum concentrations at Day 6 of dioestrus (Fig. 2). Concentrations decreased to low levels by mid to late dioestrus. Compared with normal mares, mares with endometritis had significantly lower ($P < 0{\cdot}001$) concentrations of oestrogen receptors on Day 3 of dioestrus.

Concentrations of progesterone receptors were highest during oestrus and early dioestrus and had started to decline by Day 6 of dioestrus to low levels by Day 15 of dioestrus (Fig. 3). Mares with endometritis had significantly lower ($P < 0{\cdot}001$) concentrations of progesterone receptors on Day 3 of dioestrus than did genitally normal mares.

Ovariectomized mares had concentrations of endometrial oestrogen and progesterone receptors

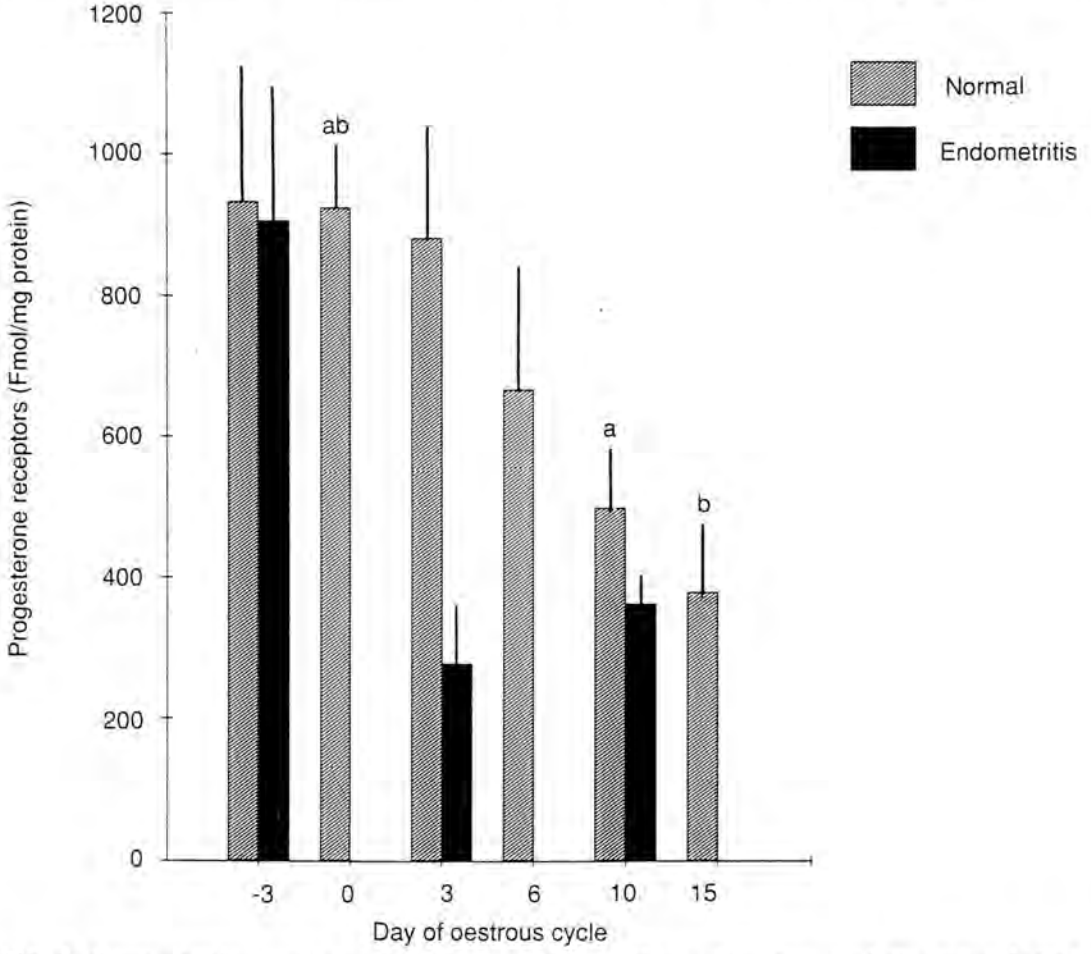

Fig. 3. Concentrations of progesterone receptors in the endometrium of the mare during the oestrous cycle. n = 3–6 per group. Day -3 = Day 3 of oestrus. Columns with similar superscripts are significantly different, $^{a,b}P < 0{\cdot}05$.

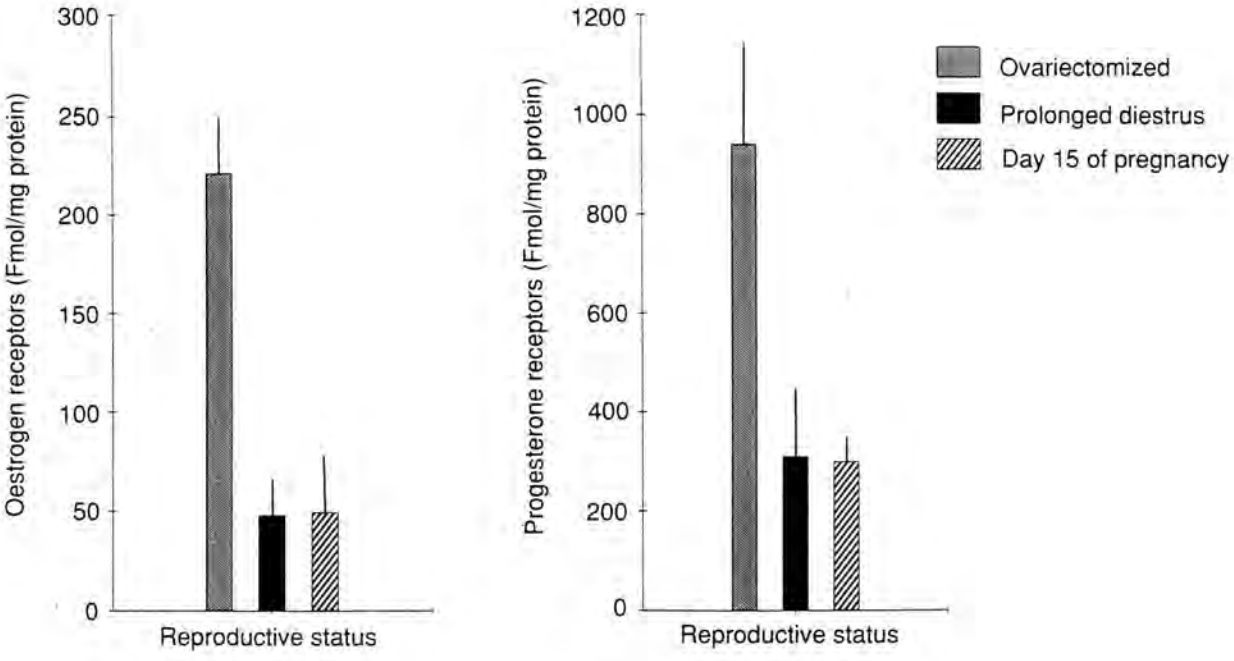

Fig. 4. Concentrations of oestrogen (left) and progesterone (right) receptors in the endometrium of ovariectomized mares (n = 5), mares at Day 15 of pregnancy (n = 6), and mares in prolonged dioestrus (n = 2).

similar to the high concentrations found in intact mares around oestrus (Fig. 4). When compared to non-pregnant mares 15 days after ovulation, mares at Day 15 of pregnancy or during prolonged dioestrus did not have significantly different concentrations of endometrial receptors.

Figure 6 shows plasma progesterone concentrations at times of endometrial biopsy specimen collection.

Discussion

Concentrations of steroid receptors in the endometrium of the mare appear to peak during oestrus and early dioestrus and decrease during mid- and late dioestrus. Patterns of receptor concentrations during the oestrous cycle in the mares studied resembled more closely those of the bitch (Johnston *et al.*, 1985; Fernandes *et al.*, 1989) and the sow (Deaver & Guthrie, 1980) than those of the cow (Vesanen *et al.*, 1988) or ewe (Koligian and Stormshak, 1977).

In the present study, assay of blood hormone concentrations showed that when progesterone concentration was low, steroid receptor concentrations were high, and when progesterone concentration increased, receptor concentrations tended to decrease. It has been reported that progesterone antagonizes the ability of oestrogen to promote the synthesis and/or replenishment of endometrial steroid receptors in other species (Clark *et al.*, 1977; Coulson & Pavlik, 1977; Evans & Leavitt, 1980). Also, it is possible that progesterone inactivates its own receptors (Walters & Clark, 1978). Further, there is evidence that the high concentrations of oestradiol present around oestrus promote synthesis of steroid receptors (Coulson & Pavlik, 1977; Evans & Leavitt, 1980; West & Brenner, 1985). Results of the present study suggest that similar relationships between hormones and their receptors may exist in the mare. Interestingly, these results support the finding in the bitch that the progesterone-dependent decrease in progesterone receptors precedes the decline in concentrations

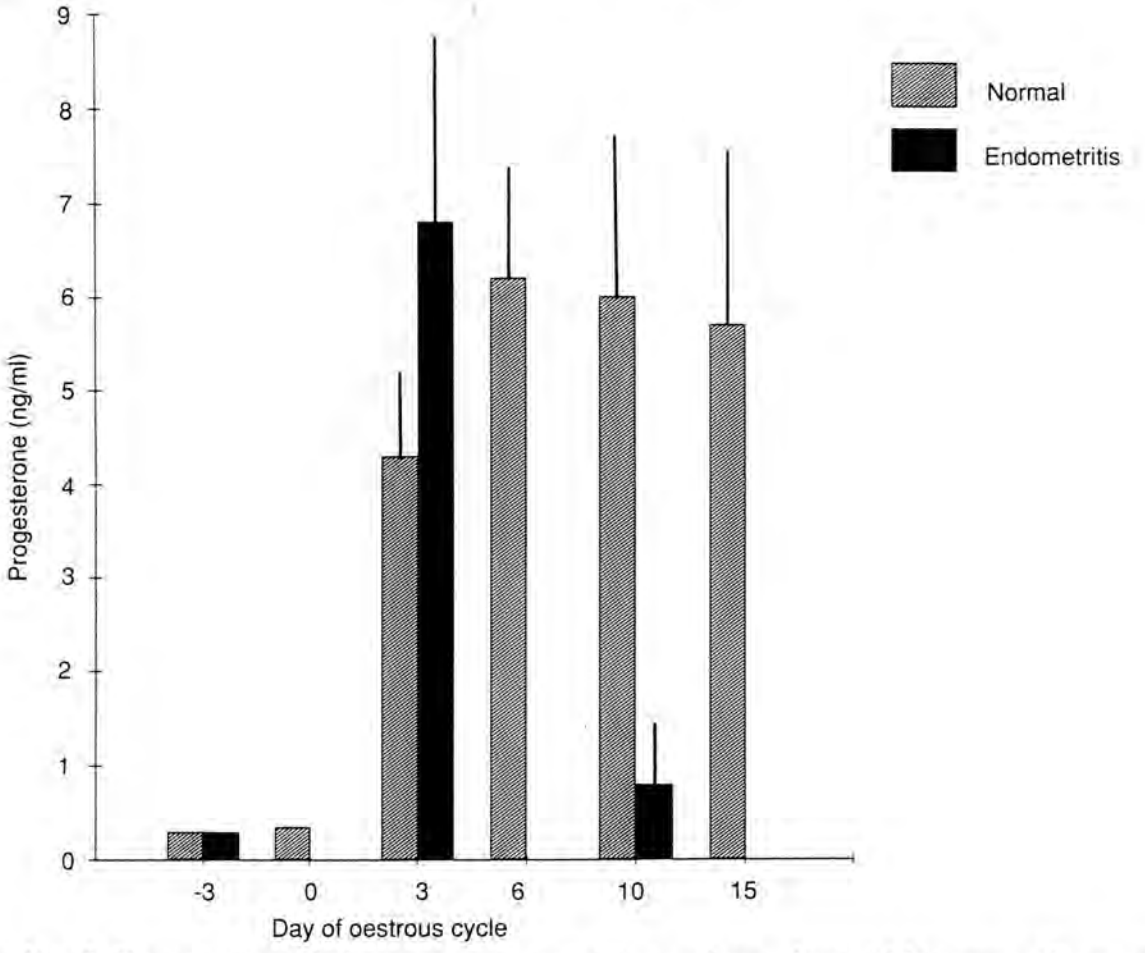

Fig. 5. Concentrations of plasma progesterone at times when endometrial biopsy samples were collected. Day -3 = Day 3 of oestrus.

of oestrogen receptors (Fernandes *et al.*, 1989).

Although our data is preliminary, it is interesting that receptor concentrations were considerably lower in mares with endometritis on Day 3 of dioestrus, than in mares with normal endometria. High oestrogen concentrations are associated with increased phagocyte function in the mare (Ganjam *et al.*, 1982). Many bacteria are introduced into the uterus at coitus. It is conceivable that lower numbers of endometrial oestrogen receptors, by leading to reduced intrauterine oestrogen concentrations during early dioestrus, might decrease uterine immune defence mechanisms and result in endometritis. Larger numbers of mares need to be studied to confirm this observation. In the present study, mares with endometritis had low concentrations of plasma progesterone by Day 10 of dioestrus because of premature luteolysis.

Mares sampled during prolonged dioestrus or during pregnancy had low concentrations of endometrial receptors that were similar to those of mares in late dioestrus. In contrast, ovariectomized mares had high concentrations of endometrial receptors, resembling peri-oestrous mares. These findings confirm the association in the mare between high circulating progesterone concentrations and low numbers of endometrial receptors.

A limitation of this study was that receptors occupied by endogenous hormone were not measured, which may explain the apparently high concentrations of endometrial receptors in ovariectomized mares. However, studies performed on uterine tissue from cows showed that less than 5% of receptor sites were occupied at any time and that receptor concentrations varied proportionately with concentrations of tissue hormones during the oestrous cycle (Senior, 1975).

Studies are in progress now to measure changes in steroid receptor numbers during various pathological conditions that affect the uterus.

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Theriogenology 38:575–580, 1992

PROGESTERONE AND ESTROGEN RECEPTOR DISTRIBUTION IN THE ENDOMETRIUM OF THE MARE

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ABSTRACT

An immunoperoxidase staining technique was used to localize receptors for progesterone and estrogen in the uterus of the mare. Specific staining for receptors was limited to cell nuclei. During estrus, stromal cells tended to stain more intensely for both receptor types than myometrial cells or luminal and glandular epithelial cells. During diestrus, staining intensities in stromal and myometrial cells tended to decrease. Staining intensities of epithelial cells were not affected by the cycle stage. Early pregnancy did not markedly affect the staining intensities of pregnant mares compared with the nonpregnant mares on Day 14 of diestrus. In mares susceptible to endometritis from which samples were taken during diestrus, stromal and myometrial staining for estrogen receptors was more intense than in endometrium from genitally-normal mares.

Key words: steroid receptors, immunocytochemistry, uterus, mare

INTRODUCTION

The uterus is able to sequester large amounts of circulating ovarian steroids due to the presence of specific high-affinity receptor proteins. Estrogen and progesterone modulate the activities of the uterus during the estrous cycle and pregnancy. Circulating concentrations of these hormones appear to regulate receptor concentrations within the endometrium of the mare. Concentrations of binding sites are highest in estrus and early diestrus and decrease later in diestrus (1). Although such steroid binding assays provide quantitative information about receptor content, they do not indicate the distribution of receptors among the constitutive types of cells of the uterus, and are prone to error due to receptor occupancy by endogenous hormones (2). The recent development of monoclonal antibodies against progesterone and estrogen receptors has allowed the use of immunocytochemical procedures in localization of receptors. These staining techniques have demonstrated a marked difference in the distribution of receptors among cell types and among species (3,4).

Acknowledgements

We thank Dr. P.G. Satyaswaroop, Hershey Medical Center, Hershey, PA, for his assistance with the progesterone receptor staining technique. We also thank Dr. P.L. Sertich and Dr. R.M. Oristaglio, University of Pennsylvania, for assistance with biopsy sample collection. Ms. S.B. Skolnik was in receipt of a Havemeyer Veterinary Student Assistantship.

We have used monoclonal antibodies to determine the cellular distribution of estrogen and progesterone receptors within the endometrium of the mare and to study whether the stage of estrous cycle, pregnancy, or the presence of endometritis affects receptor localization.

MATERIALS AND METHODS

Endometrial biopsy samples were obtained from 6 mares with Category I or IIA endometria (5) during estrus (Day 3 of estrus or on the day of ovulation) and diestrus (Day 6 or Day 10 after ovulation); from 4 mares on Day 14 of diestrus; and from 6 mares on Day 14 of pregnancy. Samples were also collected from 4 diestrous mares with persistent acute endometritis (Category III).

The biopsy samples were placed in optimal cutting temperature embedding medium[a], quick-frozen in liquid nitrogen and stored at -70°C until receptor analysis. Tissue sections were cut onto gelatin-coated slides using a cryostat microtome. The ERICA kit[b] for immunocytochemical localization of estrogen receptors in breast tumors was modified for use in uterine tissue. Briefly, the tissue was fixed in 10% formaldehyde. The primary antibody was a rat monoclonal anti-human estrogen receptor IgG and was used at a dilution of 1:10. The avidin-biotin complex staining procedure was performed according to the manufacturer's instructions, and the sections were then dehydrated and mounted. Progesterone receptors were evaluated using a mouse monoclonal anti-human progesterone receptor IgG (HPRa6) (provided by Dr P.G. Satyaswaroop, Hershey Medical Centre, Hershey, PA) at a dilution of 1:50. Cross-reactivity with the uterine progesterone receptor of the mare was confirmed by Western Blot analysis. Slides were incubated with primary antibody for 2 hours. Avidin and biotinylated peroxidase kits (Vectastain) were purchased from Vector Laboratories,[c] and the staining procedure was performed according to the manufacturer's instructions.

Control antibodies (normal rat or mouse IgG) were applied on parallel sections. Non specific binding was not detected in these sections. The rabbit uterus was run as a control in every assay. Since the rabbit uterus is relatively rich in steroid receptors, and steroid receptor distribution within the endometrium is well documented (4), staining was described as negative (0), trace, 1+= weak, 2+= moderate and 3+= strong, according to the definition of Zaino et al. (4) for the rabbit uterus.

RESULTS

All immunoperoxidase staining for estrogen and progesterone receptors was confined to cell nuclei. Specific staining of cytoplasm or endothelial cell nuclei or cytoplasm was not observed.

Intensity of staining for both estrogen and progesterone receptors varied with the type of cell. During estrus, staining for progesterone receptors was strongest in stromal cells (Table 1). The luminal and glandular epithelium stained less intensely, and myometrial cell staining was weak. By contrast, the luminal epithelium showed only weak staining for estrogen receptors (Table 2), but the degree of staining intensities in

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[a] Tissue-Tek, Miles Inc., Elkhart, IN, USA.
[b] Abbott Laboratories, Abbott Park, ILL, USA.
[c] Burlingame, CA, USA.

other tissues was similar to that for progesterone receptors. During diestrus, intensity of staining for progesterone and estrogen receptors tended to decrease in stromal cells and in the myometrium (Tables 1 and 2). In early pregnancy, staining intensity of receptors resembled that of Day 14 of diestrus. Mares with endometritis (for which samples were taken in diestrus) had intensities of nuclear staining which were similar to genitally-normal mares in mid-diestrus, except that the staining intensities for estrogen receptors in stromal and myometrial cells tended to be greater in the susceptible mares.

Table 1. Staining intensity of progesterone receptors in the mare endometrium

Horse ID	Stage of cycle	Luminal epithelium	Glandular epithelium	Endometrium: Stroma	Endometrium: Myometrium
62	estrus	2+	2+	3+	1+
104		2+	1+	3+	1+
113		3+	2+	2+	-
121		2+	2+	1+	1+
124		1+	2+	3+	1+
131		1+	1+	2+	-
62	mid-diestrus	2+	2+	2+	1+
104		2+	2+	1+	trace
113		2+	2+	3+	-
121		2+	2+	1+	1+
124		1+	1+	1+	trace
131		2+	2+	1+	1+
81	Day 14 diestrus	trace	1+	2+	trace
122		2+	2+	1+	trace
130		2+	2+	1+	1+
131		1+	1+	1+	1+
104	Day 14 pregnancy	1+	2+	2+	trace
115		3+	2+	1+	trace
117		1+	1+	2+	trace
121		2+	2+	1+	1+
122		2+	2+	1+	trace
124		2+	2+	3+	1+
Debbie	endometritis	2+	tr	1+	trace
Poona	(mid-diestrus)	2+	2+	1+	trace
Something		2+	1+	1+	trace

Staining intensities: 0=negative, 1+=weak, 2+=moderate, 3+=strong.
- = Tissue not present in sample.

Table 2. Staining intensity of estrogen receptors in the mare endometrium

Horse ID	Stage of cycle	Luminal epithelium	Glandular epithelium	Myometrium		
				Stroma		Endometrium
62	estrus	2+	2+	2+	1+	0
104		1+	2+	3+	2+	0
113		trace	2+	3+	2+	0
121		1+	3+	2+	1+	0
124		trace	1+	2+	1+	0
131		1+	2+	3+	2+	0
62	mid-diestrus	trace	1+	1+	0	0
104		1+	2+	1+	0	0
113		1+	2+	1+	0	0
121		1+	2+	1+	trace	0
124		1+	1+	1+	trace	0
131		1+	2+	1+	0	0
81	Day 14 diestrus	1+	1+	trace	0	0
122		trace	trace	trace	0	0
130		1+	1+	1+	0	0
131		1+	1+	1+	0	0
104	Day 14 pregnancy	trace	1+	1+	0	0
115		1+	1+	1+	trace	0
117		1+	trace	trace	trace	0
121		1+	1+	1+	0	0
122		1+	1+	1+	0	0
124		trace	trace	trace	trace	0
Debbie	endometritis	trace	1+	3+	1+	0
Poona	(mid-diestrus)	1+	2+	3+	1+	0
Something		1+	2+	2+	0	0
Etok		1+	2+	2+	1+	0

Staining intensities: 0=negative, 1+=weak, 2+=moderate, 3+=strong.

DISCUSSION

The present study shows that receptors for estrogen and progesterone are confined to the nuclei of uterine cells in mares. Similar results have been reported for uterine tissues of rats (6), rabbits (4), primates (7,8) and ewes (9). Species differences appear to exist in the distribution of sex steroid receptors among different cell types. In our study a rabbit uterus was used as a control in all assays, and the staining intensity of the rabbit uterus was the same as previously reported (4), but the pattern of staining in the horse uterus differed from that of the rabbit uterus. The luminal and glandular epithelium in the mare stained moderately for progesterone receptors, but only weak staining was present in these same types of cells in the rabbit uterus. In the mare uterus,

the stroma stained strongly for estrogen receptors, but in the rabbit uterus only weak staining was observed. Furthermore, the luminal epithelium of the rabbit uterus stained strongly for estrogen receptors whereas only weak staining was present in the luminal epithelium of the mare. It is perhaps not surprising that species differences exist in receptor distribution in the uterus: In these species the contrasting uterine morphologies present during the estrous cycle and the differences in uterine function during pregnancy may result from differences in sex steroid receptor concentrations. Steroid receptor concentrations have been measured in the mare uterus by steroid binding assays (1). Concentrations were highest during estrus and early diestrus and lowest in mid-diestrus. These findings are similar to those of our present study in that staining intensity in some tissues, notably the myometrium and stroma, decreased markedly during diestrus. In other species it has been shown that sex steroid receptors are up-regulated by estradiol and down-regulated by progesterone (10,11), and a similar association may exist in the mare (1).

Compared with sections of uterine tissue from diestrous mares, pregnancy did not appear to markedly alter staining intensities for estrogen and progesterone receptors. This finding is consistent with concentrations measured by steroid binding assay (1). By contrast in the ewe, estrogen receptors were suppressed during early pregnancy (9). Perhaps this result reflects the difference in timing of embryo attachment in these two species. Furthermore, in ewes the distribution of the estrogen receptor within the endometrium at the time of luteolysis and maternal recognition of pregnancy is important because of the relationship between estrogen, oxytocin and its receptors and luteolysis (9). These mechanisms still have to be investigated in detail in the mare.

There appeared to be no deficiency in sex steroid receptor densities during estrus and mid-diestrus in mares that were susceptible to persistent endometritis, and this finding confirms the results of other workers who used steroid binding assays (12). Preliminary data have shown that susceptible mares have lower uterine estrogen receptor concentrations in early diestrus, which could render them susceptible to infection introduced at coitus (1). Both the present work and that of Bracher et al. (12) studied mares in mid-diestrus; therefore, any early deficiency in estrogen receptor concentrations may have been missed.

In conclusion, we have used immunocytochemical techniques to determine the cellular distribution of sex steroid receptors in the uterus of the mare and have shown differences in receptor expression with tissue type and with the stage of the estrous cycle.

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THE EFFECT OF TRANSCERVICAL UTERINE MANIPULATIONS ON ESTABLISHMENT OF UTERINE INFECTION IN MARES UNDER THE INFLUENCE OF PROGESTERONE

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ABSTRACT

Four pony mares were used in a cross-over study to investigate the effect of different treatments on experimentally-induced endometritis. The mares were treated with progesterone to facilitate establishment of uterine infections. They received an intrauterine infusion of Streptococcus zooepidemicus 5 days after the start of progesterone therapy. Five days later, they were treated by intrauterine infusions of 2 g ampicillin in 50 ml sterile water or by sterile water without antibiotic for 3 consecutive days.

Prior to infusion of Strep. zooepidemicus, no bacteria were cultured from the uteri of the mares. However, 5 days after infusion of Strep. zooepidemicus and prior to antibiotic therapy, mixed bacterial growths were cultured from endometrial swabbings. After antibiotic therapy, ampicillin-resistant organisms were cultured from endometrial swabbings. Two other progesterone-treated mares received an intrauterine infusion of sterile phosphate buffered saline instead of bacteria. Mixed bacterial cultures were recovered 5 days later from the endometrial swabbings of these mares. It was concluded that the high circulating concentrations of progesterone were probably responsible for the treatment failure and that in clinical situations, therapy involving transcervical manipulations should not be administered when mares are in diestrus.

Key words: endometritis, progesterone, mare

INTRODUCTION

Bacterial endometritis is a primary cause of low fertility in the mare (1). Treatment of bacterial endometritis varies but often consists of large-volume uterine flushing followed by intrauterine infusion of an antibiotic, which has been selected on the basis of culture and sensitivity results (2). Mares are frequently treated when they are in estrus (3), but treatment during diestrus is also recommended (4). Intrauterine antimicrobial therapy is usually performed daily for 3 to 5 days or every other day for 2 to 5 treatments (3,5). In the present study, we report on the effect of repeated transcervical manipulations on uterine microbial populations in mares with experimentally-induced endometritis.

Acknowledgements

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[1] To whom reprint requests should be addressed.

MATERIALS AND METHODS

This study was performed as part of a larger study to evaluate inflammatory changes within the endometrium after various treatment regimens. Four pony mares with normal cyclic ovarian activity, weighing 280 to 460 kg and aged between 4 and 13 years, were randomly assigned to receive 2 treatments in a crossover study. These mares were selected on the basis of no evidence of uterine inflammation on histological examination of endometrial biopsy samples (Category 1-IIA: 6) and no bacterial growth from cultures of endometrial swabbings. All mares were judged to have good vulvar conformation, with no evidence of pneumovagina. To render the mares susceptible to endometritis, each mare received a daily intramuscular injection of 150 mg of progesterone in cottonseed oil for 16 days. This treatment regimen has been shown to induce changes in plasma hormones and in the histological appearance of the uterus consistent with diestrus (7,8).

Preparation of Bacteria for Intrauterine Infusion

Streptococcus zooepidemicus was isolated from the uterus of a mare with acute endometritis, and the bacteria were stored in 2-ml aliquots at -70°C in brain-heart infusion broth (BHIB) containing 10% glycerol. On the day before infusion, an aliquot was thawed, inoculated into 60 ml of BHIB and incubated overnight at 37°C. Bacteria were prepared for infusion by washing twice in sterile phosphate buffered saline (PBS, pH 7.2), and resuspending at $1x10^9$ colony forming units/ml by calibration, using a spectrophotometer. A sample of the inoculum was cultured to ensure that only Strep zooepidemicus was being infused into the uterus.

Infusion of Bacteria

Bacteria were infused into the uterus 5 days after the start of progesterone administration. The mare's tail was wrapped in a plastic sleeve and elevated away from the perineal region. The rectum was evacuated and the perineum cleansed thoroughly 3 times with povidone-iodine scrub. The perineum was dried between each wash. A clean plastic rectal sleeve was worn, over which a sterile surgeon's glove was placed and the hand was lubricated using a sterile water-soluble lubricant. The organisms were placed in 20 ml of sterile PBS and infused into the uterus using a sterile infusion pipette.

Two additional mares were given daily intramuscular injections of progesterone in oil. Instead of bacteria, these mares received an intrauterine infusion of 20 ml of sterile PBS (pH 7.2) 5 days after commencement of progesterone injections. These mares did not receive any of the treatments described below.

Treatments

Five days after infusion of bacteria each of the 4 mares received the following treatments in a 2 x 2 crossover study: 1) 2 g i.u. of ampicillin as sodium salt[a] in 50 ml of sterile water for injection[b]; 2) 50 ml i.u. of sterile water for injection.[b] Each treatment was administered once daily for 3 consecutive days.

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[a] Penbritin Veterinary Injectable,Smith-Kline Animal Health Ltd., Stevenage, Herts, U.K.

[b] BK Veterinary Products Ltd.,Bury St Edmonds, Suffolk, UK.

Microbiology

Endometrial swabbings were collected for microbiological culture prior to bacterial infusion (Day 5), 5 days after bacterial infusion (Day 10; just prior to administering the first treatment), and on the last day of treatment (Day 12) prior to antibiotic infusion. A final endometrial swab was obtained 4 days after progesterone treatment had ceased (Day 20). Mares with a healthy endometrium should spontaneously resolve uterine infection once progesterone administration has ceased (E.D. Watson, unpublished data). Endometrial swabbings were collected using double guarded uterine swabs.[c] All swabs were plated onto horse blood agar within 3 hours of collection and cultured aerobically for 48 hours at 37°C. Bacteria cultured were identified using standard microbiological techniques (9).

RESULTS

The results of culture of endometrial swabbings are shown in Table 1. None of the mares had evidence of uterine infection prior to infusion of bacteria or PBS. Only small volumes of fluid (<10 mm) were detected at the widest diameter of the uterine body by ultrasonography on Day 10; therefore, none of the mares were treated by lavage with large volumes of saline prior to antibiotic therapy. Only on 3 of 8 occasions the mares established a pure Strep. zooepidemicus infection; endometrial swabbings from the other mares showed mixed bacterial growth consisting mainly of coliforms with a lesser growth of Strep. zooepidemicus and Staphylococcus aureus. After treatment with ampicillin all mares remained infected. Only 1 of the mares maintained a pure streptococcal infection. In the 3 other mares there was a shift in the bacterial population post treatment. Infection was maintained in 3 mares after progesterone treatment had ceased. In these 3 mares the organisms were found to be ampicillin-resistant but sensitive to gentamicin. These mares responded to 3 consecutive daily intrauterine treatments with buffered gentamicin, and had no growth on cultures of endometrial swabbings collected 3 days after the end of treatment.

All 4 mares in the water-treatment group were infected after treatment and maintained their uterine infection after progesterone treatment had ceased. They were treated with uterine flushes on 3 consecutive days using a 0.1% povidone-iodine solution. By 3 days after the last treatment endometrial swabbings from all mares yielded no bacterial growth.

The 2 control mares which were treated with progesterone and which received sterile PBS rather than Strep. zooepidemicus had no bacterial growth from endometrial swabbings collected prior to infusion. However, post infusion, a heavy growth of coliforms (Grade 4+) was cultured from the uterus of one of the mares, and a heavy growth of Strep. zooepidemicus (Grade 4+) was cultured from the uterus of the other mare.

In all cases cytological examination of the endometrium revealing presence of inflammatory cells correlated with isolation of bacteria. These results have been reported elsewhere.[d]

DISCUSSION

In the present study experimentally-induced endometritis was used to evaluate

[c] Equivet, Suppliers: Dunwood, Bridge of Don, Aberdeenshire, U.K.
[d] McDonnell, A.M. and Watson, E.D., submitted for publication.

Table 1. Bacterial growth from endometrial swabbings collected from 4 progesterone-treated mares with experimentally-induced endometritis treated with ampicillin and water

Ampicillin treatment				
Mare	Pre-infusion of bacteria (Day 5)	Pre-treatment (Day 10)	Post-treatment (Day 12)	Post progesterone treatment (Day 20)
1	NG	MG 3+	COLI 4+	NG
2	NG	Sz 4+	MG 4+	COLI 4+
3	NG	Sz 4+	Sz 2+	COLI 3+
4	NG	MG 3+	COLI 3+	COLI 3+
Water treatment				
Mare	Pre-infusion of bacteria (Day 5)	Pre-treatment (Day 10)	Post-treatment (Day 12)	Post progesterone treatment (Day 20)
1	NG	COLI 3+	COLI 4+	COLI 3+
2	NG	Sz 3+	MG 3+	COLI 4+
3	NG	MG 2+	COLI 2+	COLI 3+
4	NG	COLI 2+	BHS/COLI 1+	MG 3+

Day 0 = Start of progesterone treatment; NG = No bacterial growth; MG = Mixed bacterial growth(ie Strep. zooepidemicus, Escherichia coli and Staphylococcus aureus); Sz = Strep. zooepidemicus; BHS = Beta haemolytic streptococci and COLI = Coliforms.
Bacterial growth was scored quantitatively [i.e., 0 to 20 colony forming units was recorded as 1+, and greater than 20 colonies as 2+, 3+ or 4+ depending on the density of growth (2+ least density, 4+ heaviest density)].

the efficacy of treatment of mares using ampicillin as the antibiotic of choice. Progesterone treatment suppresses uterine immune defense mechanisms (8) and also decreases cervical drainage (10), thereby facilitating the establishment of uterine infection.

Current therapy for endometritis is based on large-volume uterine flushing with saline or antiseptic solutions, followed by intrauterine infusion of a small volume of antibiotics selected on the basis of culture and sensitivity results (2). Ampicillin is a semisynthetic penicillin which is active against both Gram positive and Gram negative bacteria and is commonly selected for the treatment of endometritis (12). Ampicillin persists well in the uterus of the mare whether it is administered systemically (11) or by the intrauterine route (13). Concentrations of ampicillin were higher in endometrial tissue when mares were treated during diestrus than during estrus (13). Love et al (13) suggested that increased loss of intrauterine ampicillin might occur during estrus due to a patent or relaxed cervix, and could contribute to treatment failure in mares. In the present

study, although dosage, route and frequency of administration of ampicillin were used as recommended by Love et al (13), ampicillin was ineffective in eliminating the infection. Under the effects of progesterone, mares are highly susceptible to bacteria introduced during transcervical manipulation (14,15). In our study, even the 2 control mares which received an infusion of sterile PBS rather than bacteria developed an intrauterine infection. The clitoral fossa and vestibule from normal healthy mares support a large number of commensal organisms such as Strep. zooepidemicus and E. coli (15). In our study, all procedures which involved entering the uterus were carried out as aseptically as possible; however, it was demonstrated that normal commensals of the anterior vagina were inadvertently introduced during transcervical manipulation, as in endometrial swabbing or intrauterine infusion of antibiotics. We have shown that daily intrauterine infusion of antibiotics significantly increased the risk of introduction of opportunist pathogens, but even placement of an indwelling uterine catheter during diestrus for administration of treatment may introduce sufficient opportunistic pathogens to result in a mixed uterine infection, which may outweigh the benefits of therapy during diestrus.

In our study it was also interesting to note that apart from 1 mare, none had cleared the uterine infection by 4 days after progesterone administration had ceased. All mares had received a luteolytic dose of a prostaglandin $F_{2\alpha}$ analog 1 day after the cessation of progesterone treatment, to ensure that any endogenous source of progesterone, i.e., a corpus luteum, had been eliminated. Perhaps the length of time that the mares were maintained on progesterone had a residual effect on uterine immune defense mechanisms. All mares responded to appropriate antibiotic or antiseptic therapy based on culture and sensitivity results, and subsequently cleared the infection. Thus treatment was successful after progesterone was withdrawn. It is also likely that the mares were coming into estrus at this time, which helped in the elimination of infection.

In normally cycling mares, treatment involving transcervical manipulations during diestrus can result in shortening of the cycle, with premature return to estrus (16). More importantly, however, infusion of small volumes of antibiotic resulted in prolongation of diestrus in about 50% of cases, which would increase the length of time the uterus was under the influence of progesterone (17). It would have been interesting to study the efficacy of treating these mares during a period of estrogen treatment. However, we have shown previously that genitally-normal mares will not retain a uterine infection for more than 18 hours when they are being maintained on estrus levels of estradiol (18). We conclude that transcervical manipulation during periods of high circulating concentrations of progesterone carries a high risk of introduction of opportunistic pathogens.

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EQUINE VETERINARY EDUCATION
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Tutorial Article

Uterine inflammation after intrauterine infusion with a low volume of Utrin

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Introduction

It is common practice to treat mares with persistent mating-induced endometritis (PMIE) or chronic uterine infection after mating with a combination of uterine lavage and/or i.v. oxytocin followed by intrauterine infusion of antibiotic (LeBlanc 1994; Pycock and Newcombe 1996; Troedsson 1997). Because of the tendency for such mares to accumulate intrauterine fluid, it has been suggested that antibiotic should be infused in a low volume after oxytocin therapy to evacuate the uterus. Utrin[1] in combination with crystalline penicillin is a commonly used antibiotic cocktail for uterine infusion in the UK. The manufacturers recommend dilution to 100 ml with sterile water, and high pregnancy rates have been reported after its use (Ricketts 1995, 1997). However, in some reports, a volume of 20 ml has been used (Pycock 1994; Pycock and Newcombe 1996).

This report concerns the study of a potential precipitant reaction in using combined antibiotic preparations in the treatment of the mare's uterus.

Clinical findings

A 17-year-old Thoroughbred mare with a history of subfertility was presented in 1996 for artificial insemination. The mare had a sloping vulva which required Caslick's vulvoplasty. Aerobic culture and cytological examination of a bacteriological swab collected from the uterus were negative. When the mare was in oestrus with a 35 mm ovarian follicle, she was treated with human chorionic gonadotrophin (hCG) (2500 iu). On the next day she was inseminated with transported chilled semen and by the following day she had ovulated. The mare had a small pocket of uterine fluid after insemination and was treated with 25 iu oxytocin i.v. One hour later Utrin[1] dissolved in 15 ml sterile water for injection was infused into the uterus, followed by 5 mega benzylpenicillin dissolved in 10 ml sterile water. This treatment was repeated daily for another 3 days. By Day 5 after ovulation, no intrauterine fluid was detected on ultrasonography. By 6 days after insemination (5 or 6 days after ovulation) a dense mass was seen within the uterine lumen (**Fig 1**). It was suspected that this may have been a deposit from the intrauterine infusions. No uterine fluid was present. It was decided to leave the mare rather than perform a uterine flush as the embryo reaches the uterus on Days 5 or 6. On Day 14 no conceptus was present upon ultrasonography. By the time of this scan the mass was not obvious and the uterus was free of fluid. The mare was treated with prostaglandin $F_{2\alpha}$ and reinseminated 8 days later. On the day after AI some uterine fluid was present and the uterus was flushed with sterile buffered saline (pH 7.0). The recovered fluid was very cloudy and contained clots of yellow material. Cytology was performed on the recovered flush and many neutrophils were seen surrounding crystals (**Fig 2**). Flushes were continued daily until the recovered fluid was clear. The mare was not pregnant to this insemination and was not reinseminated.

Because of the problems encountered with this mare, Utrin[1] and benzylpenicillin (Crystapen)[2] were mixed in a tube to test the solubility. **Figure 3** shows 5 mega Crystapen[2] in 10 ml water being added to Utrin[1] (1 vial in 15 ml water). A sticky precipitate formed immediately which sedimented out 24 h later. By contrast, when the volume was 100 ml, a clear solution was formed with no visible precipitate. In order to investigate the uterine response to infusion of a low volume of Crystapen[2] and Utrin[1], 3 pony mares received an intrauterine infusion of Utrin[1] dissolved in 10 ml sterile water followed by Crystapen[2] dissolved in 10 ml sterile water immediately

TABLE 1: Differential cell counts of uterine lavage fluid before and 5 days after intrauterine infusion of 10 ml Utrin followed by 10 ml Crystapen

Pony	Neutrophils (%)		Macrophages (%)		Epithelial cells (%)		Other (%)*	
	Before	After	Before	After	Before	After	Before	After
1	14	31	2	40	82	27	2	2
2	29	59	4	35	66	4	1	2
3	45	26	6	61	45	9	4	4

*Other cells were lymphocytes and eosinophils.

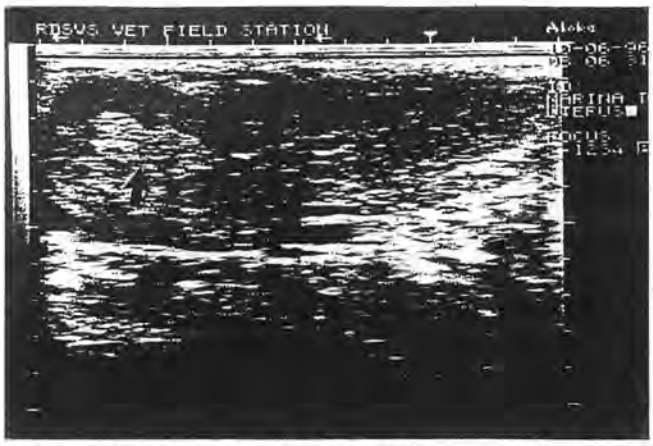

Fig 1: Ultrasound scan of the mare's uterus 2 days after cessation of treatment of intrauterine infusion of Utrin in 15 ml water followed by Crystapen in 10 ml water for injection. *Note the echogenic material in the lumen on the left screen (arrow).*

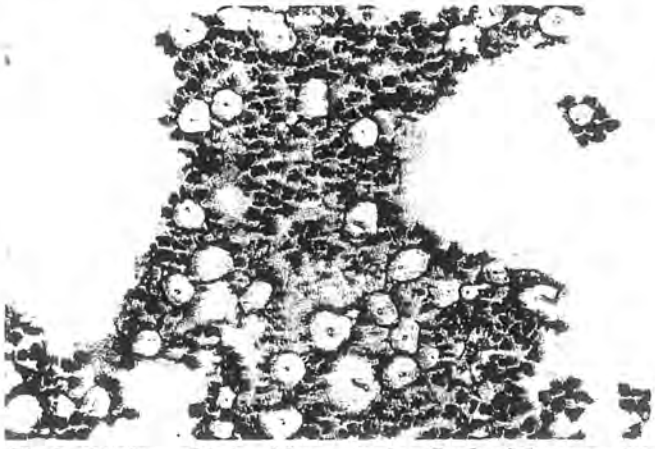

Fig 2: Cytology obtained from uterine flush of the mare 20 days after treatment. *Stained with Diff-Quik.* ***Note*** *the presence of crystals surrounded by neutrophils (x40).*

after a uterine flush with 50 ml sterile buffered saline (pH 7.0). *Mares 1* and *2* were ovariectomised, which in itself does not compromise uterine defence mechanisms (Watson 1986) and *Mare 3* was treated on the day of ovulation. All of these mares already had a mild degree of endometritis based on having more than 5% neutrophils on cytological examination of their endometrium, but had no intrauterine fluid visible on ultrasound scan. Five days later a second 50 ml uterine flush was performed. **Figure 4** shows a cytospin preparation of the recovered flush. Cytology revealed the presence of many macrophages together with some neutrophils. Percentage of inflammatory cells increased substantially in all ponies after infusion (**Table 1**). *Mares 1* and *2* had a small pocket of uterine fluid after treatment which resolved by Day 4 or 5 post-treatment, but *Mare 3* accumulated large volumes of uterine fluid (**Fig 5**) which persisted until after the second uterine flush.

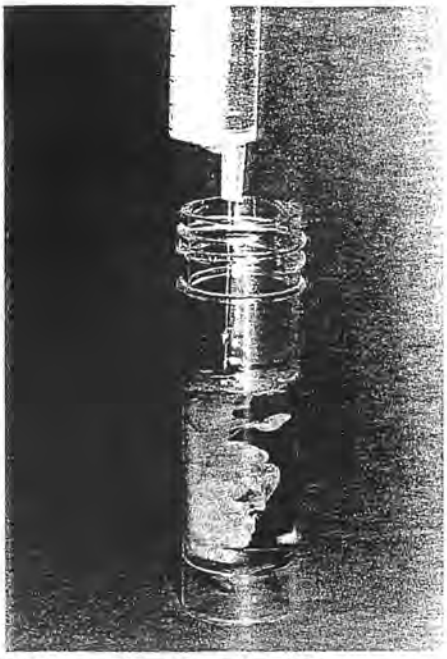

Fig 3a: Addition of Crystapen to Utrin.

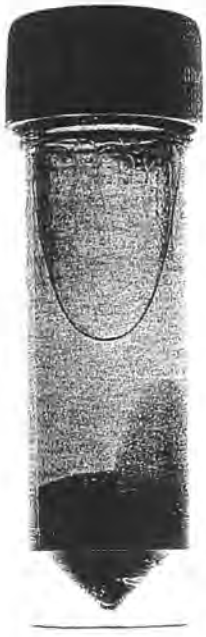

Fig 3b: Sediment 24 h later.

Discussion

The observations in the present paper show clearl infusion of a small volume of Utrin[1] in combinatio Crystapen[2], which would normally be acceptable with antibiotic preparations, is not satisfactory with particular antibiotic combination. When resuspende large volume (100 ml), as recommended b manufacturers, a clear solution results which would

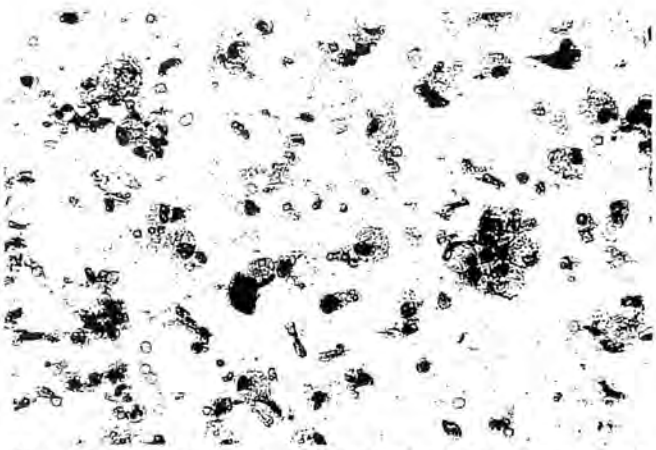

Fig 4: Cytology from uterine flush obtained after a single intrauterine treatment (x 25). Note the presence of many macrophages.

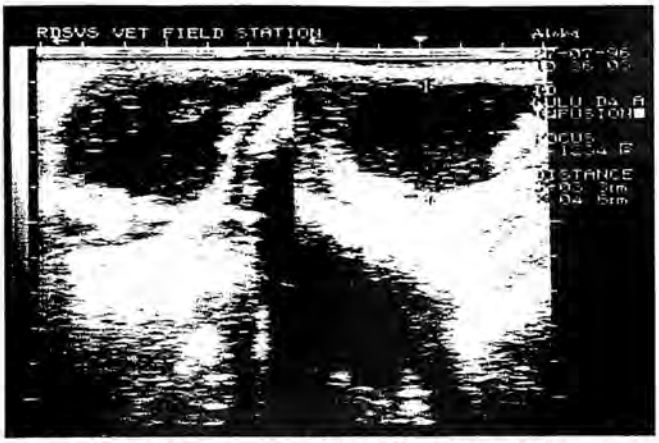

Fig 5: Ultrasound scan of Mare 3's *uterus 4 days after single uterine infusion of Utrin (10 ml) followed by Crystapen (10 ml).*

anticipated to cause irritation in the mare's uterus. However, smaller infusion volumes are preferable in mares with uterine clearance problems. In a 20–25 ml volume an insoluble precipitate was formed after mixing Crystapen[2] and Utrin[1] which clearly irritated the mare's endometrium as shown by the inflammatory cells present on cytological examination of a uterine flush. Although high pregnancy rates have been reported using this combination (Pycock and Newcombe 1996), it appeared that these authors would normally use this treatment only once after breeding. The present study shows that repeated treatments with small volumes of Utrin[1] and Crystapen[2] cannot be recommended and the degree of irritation produced may well predispose the mare to problems such as secondary mycotic infections. It should also be considered that the mares in our study were prone to uterine inflammation and it is probable that genitally normal mares with good uterine clearance would be better able to resolve the result of repeated infusions. Consultation with the manufacturers confirmed that it is a recognised problem that addition of penicillin to Utrin[1] will cause precipitation of salts if volumes are reduced below 100 ml (P. Stanford, Vetoquinol, personal communication). It is assumed that veterinarians using small volumes are not mixing the drugs prior to infusion, and are therefore unaware of the precipitation reaction. In the clinical situation, however, mares with normal uteri should not be treated with antibiotics. In our clinic we are now tending also not to infuse antibiotics in mares with PMIE unless there is bacteriological evidence that the mare is infected. Rather, mares accumulating a large amount of fluid are treated with early uterine lavage within 12 h of mating followed by multiple daily injections of oxytocin, combined with exercise. Those with small volumes of fluid are treated only with oxytocin. Mares with PMIE often do not have intrauterine bacterial infection by 48 h after insemination (Nikolakopoulos and Watson 1997) and even experimental administration of clenbuterol (a uterine relaxant) to induce susceptibility to endometritis, frequently does not predispose mares to persistent uterine infection after infusion of *Streptococcus zooepidemicus* on the day of ovulation (Nikolakopoulos and Watson 1999). Recent reports from other centres would support our current treatment regimen (LeBlanc 1997; Troedsson 1997).

Manufacturers' addresses

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[2]Schering Plough Animal Health, Uxbridge, Middlesex, UK.

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